THE ROLE OF THE MURINE HOMEOBOX GENE CUX-1 IN KIDNEY DEVELOPMENT AND POLYCYSTIC KIDNEY DISEASE

By

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“In Kansas we are only restrained by the limits of our dreams.”

-Jared Grantham, M.D.,
University Distinguished Professor of Medicine.
Quote taken from his Harry Statland Professorship in Nephrology Investititure
Speech, December 8, 2005

FOR SILVIO

Now cracks a noble heart. Good-night, sweet prince,
may flights of angels sing thee to thy rest!
-***Hamlet*** **(5.2.304)**

I dedicate this piece of work in memory of my father who was a victim to the horrors of Polycystic Kidney Disease.

ACKNOWLEDGEMENTS

I would first like to express my deepest appreciation to my advisor Dr. Gregory B. Vanden Heuvel Ph.D. His constant guidance, support and belief in me has helped me to become the scientist I am today. I am very grateful for the opportunities he has provided me during my graduate career at the University of Kansas Medical Center. He introduced me to Developmental Biology and has helped me to realize my dream of studying science at a high level. I will forever be thankful for the significant impact he has had on my life and his help to ensure that my career progressed accordingly. It has been a special privilege being his first Ph.D. student. Finally, I would like to say that I am very thankful that I have been a part of his lab and working on such an outstanding project to determine if Cux-1 is involved in polycystic kidney disease and laying down the foundations for his lab to elucidate future potential mechanism(s) of Cux-1 function in polycystic kidney disease.

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to call them my family. Working with them over the past 7 years has been a tremendous privilege and I will always remember them.

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I must now express my appreciation to my closest friends and family without which nothing would be possible. I would like to thank my closest and best friend Dr. Justin Thomas D.O., for all of our stimulating philosophical discussions and his support throughout these challenging years. He was with me during my graduate work at the University of Florida and has been consistently supportive of my "unique path" the whole time even after I moved to Kansas. I would also like to thank my best friend from childhood, Vineet Chawla, J.D., for his consistent friendship, support and encouragement throughout my life. I consider both of these men to be my brothers.

There are really no words I can say to describe my appreciation to my parents without which none of this would be possible. Any success I have is only the reflection of their success as parents. My mother's support has been the backbone for my life especially during the 9 years that I have collectively spent in graduate school. My father was my inspiration as he encouraged me to dream and question everything. Most importantly he taught me to be very adaptable and to be a survivor, all characteristics that I think are critical for any good scientist. Sadly and unfortunately, my father Silvio Alcalay is no longer with us as he suffered from Polycystic Kidney Disease, which resulted in his death. It is with this body of work and my future endeavors that I honor his life and his memory. I hope that my unique characteristics and values as a man will help to make the world a better place and that my contributions in science may help one day to result or lead to the further knowledge of and treatment for polycystic kidney disease.

ABSTRACT

THE ROLE OF THE MURINE HOMEOBOX GENE CUX-1 IN KIDNEY DEVELOPMENT AND POLYCYSTIC KIDNEY DISEASE

The murine homeobox gene *cux-1* is evolutionarily conserved in drosophila, mice and humans. Cux-1 contains four DNA binding domains (3 cut repeat domains and a homeodomain) and functions as a transcription factor that represses the expression of the cyclin kinase inhibitor p27 during S-phase of the cell-cycle. Cux-1 is highly expressed in proliferating cells within the nephrogenic zone of developing kidneys. The role of Cux-1 during kidney development and Polycystic Kidney Disease (PKD) is unclear.

Cux-1 is a transcription factor that binds to DNA when it is dephosphorylated. Calcineurin A (CnA) is a phosphatase that might be involved in regulating Cux-1 as both are expressed during early kidney development. Previous studies demonstrated that CnA knockout (-/-) mice display renal hypoplasia associated with ectopic expression of p27 in the nephrogenic zone. The opposite phenotype was observed when Cux-1 is overexpressed. Therefore metanephric kidney cultures, overexpressing Cux-1 were grown in the presence of cyclosporine A to inhibit Calcineurin. Overexpression of Cux-1 rescued growth inhibition due to Calcineurin inhibition. Calcineurin inhibition resulted in increased phospho-Cux-1 levels suggesting that Calcineurin may regulate Cux-1 and thus revealing a new pathway in kidney development.

The *cpk* mouse model is the most widely characterized model for PKD. A hallmark of PKD is increased cell proliferation. The mechanism of cell proliferation in PKD is unclear although deregulation of cyclin kinase inhibitors appears to be involved. Cux-1 is highly expressed in *cpk* kidneys, however it is unclear if Cux-1 is required for PKD. The results here demonstrate that a mutation of Cux-1 (*cux-1ΔCR1*) which lacks a Cathepsin-L proteolytic cleavage site, results in severe PKD when crossed onto cpk mice. Upregulation of Cux-1ΔCR1 was observed and correlated with attenuated levels of p27 within *cpk* kidneys which suggests a potential mechanism for the acceleration of PKD. Alteration of the PKD phenotype by Cux-1 suggests that Cux-1 may act as a candidate modifier gene of PKD. The collection of studies presented within this body of work has helped to elucidate the importance of Cux-1 regulation by post-translational modification which requires further investigation as a critical factor in kidney development and PKD.

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CHAPTER ONE

BACKGROUND

The Drosophila cut gene

Drosophila melanogaster has served as a convenient model for identifying genes and genetic interactions in the 20th century. Through the use of "gain of function" mutations, e.g. the conversion of antenna to an ectopic pair of second thoracic legs in the mutation *Antennapedia*, and "loss-of-function" mutations, insights have been gained into molecular mechanisms of Drosophila development. The Drosophila *cut* locus (1) is highly susceptible to mutation and is a region of genetic instability (2). The *cut* locus was given its name from studies where mutations within the locus lead to loss of wing tissue resulting in an easily distinguishable "cut wing" phenotype (3). Ectopic expression of Cut or the expression of an evolutionarily conserved mammalian homologue rescues the Cut wing mutant phenotype (4, 5). This demonstrated the requirement for *cut* expression in wing development. Several *cut* mutations have been characterized since 1925 that cause developmental defects in drosophila wings, legs, external sensory organs, Malpighian tubules, tracheal system and within the central nervous system (6-8). Mutation within the *cut* locus can be termed as viable or lethal mutation (**Table 1-1**). *cut* locus mutations are divided into five categories based upon the tissues affected, viability, location within the locus and complementation with other *cut* mutations as presented (3). There are three types of lethal mutations resulting in the failure of the

embryo to develop, termed Lethal I, Lethal II and Lethal III (See **Table 1-1**). A mutation is considered viable if embryonic lethality does not occur as a result of mutation. *Kinked femur* (kf) and *cut wing* (ct), are examples of viable cut mutations which lead to limb malformations in the leg and wing, respectively (9,10). Viable mutations are similarly characterized by the loss of Cut expression. Cut is highly expressed in exclusive regions within the Peripheral Nervous System (PNS), the Central Nervous System (CNS), Malphigian Tubules (the insect functional kidney), tracheal histoblasts, cells surrounding the spiracles, and adipose cells (11,12). Therefore, Cut expression is considered to be critical for embryogenesis and organ morphogenesis during fly development.

Table 1-1. Classification of cut gene locus mutations

Type of cut mutation:	Kf	ct	Lethal type I	Lethal type II	Lethal type III
	Kinked Wing Femur	cut	Malpighian tubules Spiracles ES organs CNS Larval lethality Vibrissae Cut wing	Kinked femur Malpighian tubules Spiracles ES organs CNS Larval lethality Vibrissae Cut wing	Larval lethality Vibrissae Cut wing

Table 1-1. Mutations within the drosophila cut locus have been classified within 5 categories which include Kf, ct, Lethal type I, Lethal type II, and Lethal type III. Regions which are defective upon incurring a Kf, ct, Lethal type I, Lethal type II and Lethal type III mutation are listed in the above table. Modified from Nepveu et al. (13)

Genetic studies conducted in drosophila suggest that *cut* may play an important role in determining cell-type specificity within the PNS, CNS, and Malphigian Tubules. This was supported by the observation that *cut* is expressed within developing precursor cells corresponding to different cell types within the PNS, CNS and Malphigian Tubules before these cells undergo different developmental programs (11). This pattern of expression suggests that cut plays a role in determining cell-type specificity. The *cut* gene is also involved in development of the fly Antennae (14). Tissue specific expression of *cut* is directed by enhancers located upstream of the gene (3). Defects within organ tissue caused by *cut* mutations may be the result of cells enrolled in the wrong developmental program (6, 12). For example, embryonic lethal *cut* mutations caused the transformation of external sensory (es) organs into internal (chordotonal) sensory organs (12). In addition, there was the observation that *cut* mutations convert the cells of the Malpighian tubules into gut cells, suggesting that *cut* is necessary for cell type specification and morphogenesis occurring during Malpighian tubule development (15). Moreover, in *cut wing* mutants, loss of *cut* expression in the presumptive wing margin results in the loss of some adjacent epithelial cells (probably by apoptosis), thereby producing the truncated wing phenotype (4,5). Collectively, when all of these observations are considered together it is reasonable to consider the drosophila *cut* gene as a determinant of cell type specificity.

Since when the "Cut wing" phenotype was first identified in Drosophila , numerous mutations associated with the partial or complete loss of cut expression

have been identified in Drosophila (2-14). **Table 1-1** shows a list of Drosophila mutations that lead to lethality with organ defects. Our laboratory has been particularly interested in the *cut* mutation associated with the loss of the Malphigian tubules, the insect organ that functionally resembles a kidney (9,10). Using this information we, and others, have been able to identify a role for one of the mammalian Cut proteins, Cux-1, in the regulation of kidney development (15-20).

Cut homologues

Studies in Drosophila have led to the discovery of genes, termed homeotic genes, which regulate development. Ectopic expression of homeotic genes, can lead to developmental malformations that disturb normal development (21, 22). Homeotic genes act to control complex developmental pathways by regulating gene expression. Many of the developmental control genes contain a "homeobox", a homologous 180-bp nucleotide sequence of DNA conserved across species. The homeobox encodes a 60 amino acid DNA binding domain motif called a homeodomain that characterizes this family of transcription factors. Homeobox genes have been shown to regulate organogenesis and cell proliferation, pattern segment identity, and determine anterior-posterior axes and cell fate decisions during embryogenesis (16, 23,24). Homeobox genes can be divided into two broad classes: *hox* genes and divergent homeobox genes. *hox* genes are located in clusters whereas divergent homeobox genes are located in discrete positions throughout the genome.

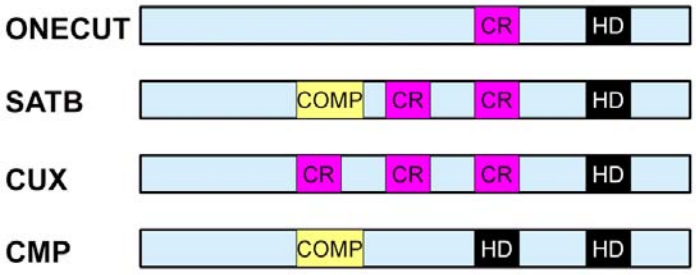


Figure 1-1. The Cut superclass family of homeodomain proteins. The Cut superclass homeodomain transcription factor family tree consists of the ONECUT family members, the SATB family members, the CUX family members, and the CMP family members. Motifs include cut repeats (CR), homeodomain (HD) and Compass domain (COMP)**.** Image taken from Alcalay et al. (241).

One member of the divergent homeobox gene family is the *cut* superclass (**Figure 1-1**). The cut superclass of homeobox genes is comprised of four classes: "ONECUT" (with 1 cut domain and 1 homeodomain), "SATB" special AT rich binding protein (with 1 "COMPASS" domain, 2 cut domains, and 1 homeodomain), "Cux" (with 3 cut domains and 1 homeodomain), and "CMP" (1 Compass domain and 2 homeodomains) (25). A comprehensive survey of the cut superclass genes were evaluated in different species. (71) Burglin and Cassata (25) reported that there are only two cut domain containing genes in Drosophila, one CUX and one ONECUT type. A neuron specific ONECUT homeobox gene was isolated in zebrafish with a cut domain that shares 88% amino acid identity with drosophila ONECUT (26). Caenorhabditis elegans has undergone an expansion of the ONECUT subclass genes and has a gene cluster with three ONECUT class genes, one of which, the CMP family, has lost the cut domain (25). Two of these genes contain a

conserved sequence motif, termed OCAM, which seems to be nematode specific. A recently uncovered C. elegans CUX gene has sequence conservation in its amino-terminus with vertebrate CUX proteins. Since a homologous cut cDNA sequence was reported in C. Elegans but not in yeast, Cut proteins appear to be conserved amongst metazoans. As represented in **Figure 1-2**, Cux protein homologues have been identified in a number of species including: human (CDP) (27), Dog (Clox) (28), rat (CDP2) (29), drosophila (Cut) (1) and mouse (Cux-1) (19). While these homologues all contain a homeodomain and 3 cut repeats, there are several truncated Cut proteins that have been identified which include: testis Cux-1 (30), CASP (31) and Cux-1 P75 (32), Cux-1 P90 (33) and Cux-1 P110 (32). In addition, there are other proteins that contain Cut repeats and a homeodomain such as Hepatocyte nuclear factor 6 (HNF6) (34), OC-2 (35), and SATB1 (36). To simplify the terminology, the term "*cux-1*" or "Cux-1" will be referred throughout the dissertation to represent all homologous forms of the Cux-1 gene and protein respectively.

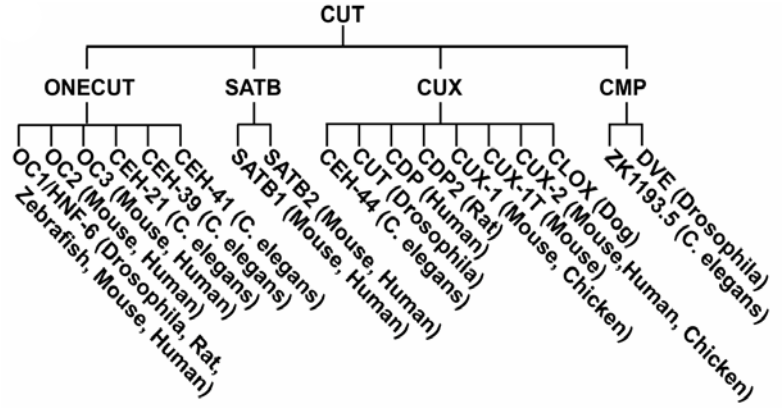


Figure 1-2. Cut superclass of homeobox genes are evolutionarily conserved between species. There is evolutionary conservation between species of CUT homologues within ONECUT, SATB, CUX and CMP CUT family members.

Murine models for Cux-1

There have been 5 mouse models of Cux-1 that have been generated to date: Cux-1ΔCR1 (37), Cux-1ΔC/ΔHD (38, 39), CUTL1^{laCZ} (40), Cux-1 p75 transgenic (41), and Cut CMV/Cux-1 Transgenic (20) (**Figure 1-3**). Tufarelli et al. (37), designed a targeting construct to replace the first cut repeat (CR1) with a neomycin resistance cassette. Their goal was to introduce a nonsense mutation after position 1319 of the 4.5-kb reading frame of Cux-1. They expected to generate a truncated product of ~60 kDa with this construct. In contrast, mice expressed a mutant form of the protein, with an internal deletion of 246 amino acids encompassing the first cut repeat domain. The CR2, CR3, HD and C-tail of Cux-1 remained intact. They discovered that an exon skipping event had occurred resulting in the removal of exons 10 and 11. The deletion, termed *cux-1ΔCR1*, results in mice displaying a mild phenotype which is characterized by curly vibrissae and wavy hair. Tufarelli et al.(37) observed that offspring of homozygote Cux-1ΔCR1 females had a high degree of mortality. There was no correlation between the genotype of the pups and their mortality.

Animal lethality was rescued by fostering litters of mutant females to lactating CD1 females. Tufarelli et al. (37) found that at day 3 of lactation, the milk of homozygous mutant females had decreased levels of ϵ-Casein, but by day 8 the levels of ϵ-Casein were the same as in the wild-type milk. However, since only a decrease in one subpopulation of caseins was observed, this result could not explain the high degree of lethality. Histological analysis revealed no observable differences

between wild-type and mutant mammary glands. Since a structural defect in mutant mammary glands was not evident, the authors proposed that the impaired lactation defect might be a result of decreased milk production, production of milk with altered properties, behavioral changes, or a combination of these factors. The cause of the lactation defect in *cux-1ΔCR1* mutant mice is currently not known. The distribution of Cux-1ΔCR1 mutant protein in mutant mice is similar to that of wild-type. However, the Cux-1ΔCR1 protein is expressed in much higher levels than wild-type Cux-1 protein in spleen and thymus. The Cux-1ΔCR1 protein appears to bind very tightly to DNA as Cux-1ΔCR1 is less readily competed off with unlabeled oligonucleotide compared to wild type Cux-1.

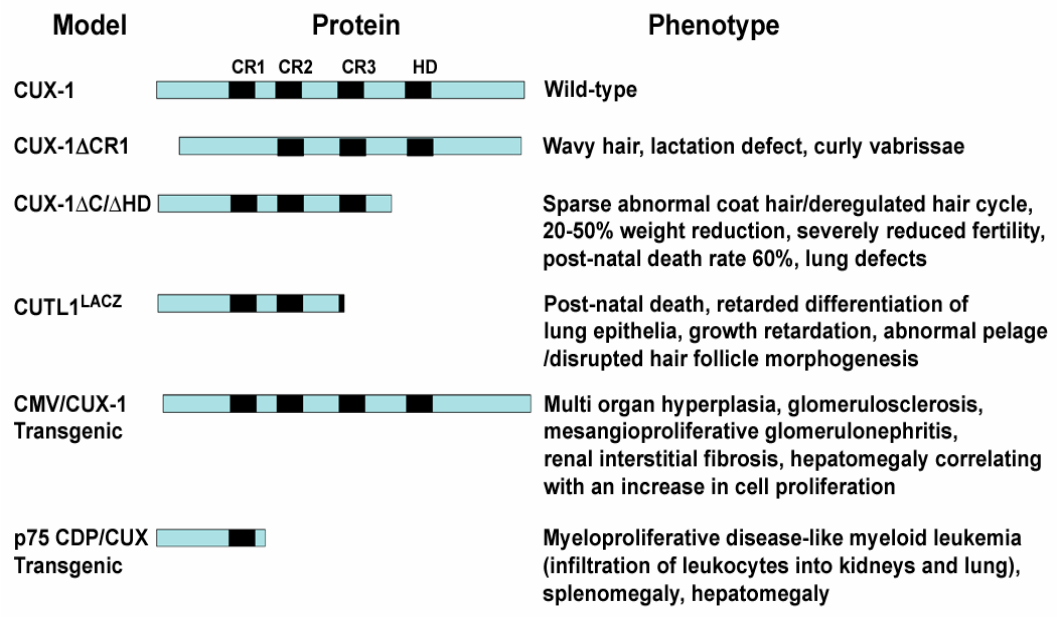


Figure 1-3. Murine animal models used to study the homeobox gene Cux-1. Image taken from Alcalay et al. (241).

A targeting construct was generated for the purpose of deleting a 4kb region encompassing the HD within exons 20 and 21 of cux-1 coding sequence. A stop codon within exon 20, which encodes the beginning of the homeodomain, was introduced to force premature translational termination. This deletion of the C-terminus of Cux-1 includes the homeodomain while retaining CR1, CR2 and CR3 domains. It results in mice with a more severe phenotype than *cux-1ΔCR1* mutant mice. The mutation, termed *cux-1ΔC/ΔHD*, (38, 39) results in stunted growth. Homozygous mutant *cux-1ΔC/ΔHD* pups have a similar appearance to their littermates at birth. The *cux-1ΔC/ΔHD* pups nursed normally, and milk was observed within their stomachs during the first couple of days after birth, but they failed to thrive. By the end of the first post-natal week of life, many *cux-1ΔC/ΔHD* pups were smaller than their littermates. The *cux-1ΔC/ΔHD* runted phenotype may be a result of defective nutrient absorption within the gastrointestinal tract. However, histological sections stained with hematoxylin and eosin revealed no structural abnormalities in the small intestine of mutant mice. Moreover, no abnormalities were observed when other organs of the gastrointestinal (GI) tract were examined, such as the stomach and the colon. Therefore, the authors concluded that the *cux-1ΔC/ΔHD* mutation did not affect development of these organs. It is still not clear as to how *cux-1ΔC/ΔHD* animals have a runt phenotype.

Most of the *cux-1ΔC/ΔHD* pups die within the first 10 days of postnatal life. Greater than 70% of *cux-1ΔC/ΔHD* pups failed to survive to weaning age. Therefore, in addition to severe weight loss, a high postnatal mortality rate of 60% to 70% was

observed. The homozygous mutant mice that do survive to adulthood have a normal life span but are severely growth retarded and weigh 30 to 50% less than their normal littermates. *cux-1ΔC/ΔHD* mice are born at mendelian frequency. They appear to die after birth as a result of a defect in lung development characterized by delayed differentiation of lung epithelia. These *in vivo* results appear to have revealed a role for Cux-1 in lung maturation. *cux-1ΔC/ΔHD* hetereozygotes have a normal phenotype in that they have a normal appearance and are fertile. In contrast, *cux-1ΔC/ΔHD* mice have abnormal coat hair with males having severely reduced fertility. There was no significant difference in wild-type, hetereozygote and *cux-1ΔC/ΔHD* homozygote testes weight. Histological and gross examination revealed that the testes and epididymis were normal in mutants. However, serum testosterone levels in mice heterozygote or homozygote for *cux-1ΔC/ΔHD* were significantly lower than those in wild-type male littermates. Yet, the lower testosterone levels did not correlate to reduced male fertility in *cux-1ΔC/ΔHD* hetereozygotes. Therefore reduced testosterone levels may not be involved in the reduced male fertility phenotype. The mechanism for reduced fertility in *cux-1ΔC/ΔHD* male mice is unknown.

Luong et al.(38) suggested that the deregulated hair cycle and severely diminished fertility implies that Cux-1 may be required for dermal and reproductive development. In yet another very interesting finding, histopathological examination on *cux-1ΔC/ΔHD* bone marrow and sternebrae revealed hyperplasia of myeloid cell types (39). In contrast to myeloid hyperplasia *cux-1ΔC/ΔHD* mice had reduced

thymic cellularity due to enhanced apoptosis, with loss of CD4(+)CD8(+) thymocytes. However, in maturing thymocytes, CD25 was ectopically expressed.

Tumor necrosis factor (TNF) was elevated in thymus of mutant mice which may suggest that TNF is a target gene for Cux-1 mediated repression. There was a 2- to 3-fold reduction in total bone marrow B-lineage due to enhanced apoptosis. The authors suggested that the observed immunological phenotypes were a result of underexpression of survival factors or overexpression of death-inducing factors. So Cux-1 may function to regulate normal hematopoiesis, presumably by modulating the expression levels of survival and/or apoptosis factors within a given microenvironment. However, the mechanism behind these observations is still not clear. Hematoxylin-and-eosin-stained sections did not reveal histological defects in the major internal organs or the brains of *cux-1ΔC/ΔHD* mice. *cux-1ΔC/ΔHD* homozygous mutant mice are very susceptible to bacterial infections and have purulent rhinitis characterized by mucosal and submucosal purulent infiltrates within the nasal turbinates (39).

cux-1ΔC/ΔHD pups initiate hair shedding during the 2nd to 3rd post-natal week. At the end of the first post-natal month, *cux-1ΔC/ΔHD* mice are completely bald except for very thin hair covering parts of the ventral region and head (38, 39). The coat hair will eventually grow back over a period of several months, with regrowth being more evident in female than in male *cux-1ΔC/ΔHD* mice. The hair that is regrown has a distinctively light gray color and often appears longer than the hair of wild-type animals. *cux-1ΔC/ΔHD* mutant mice have scant and distorted hair

that appears to be slender and broken off in contrast to wild-type mice where there was a thick mat of normal hair. *cux-1ΔC/ΔHD* mutant hair had a variety of deformities that included kinky, twisted and flattened characteristics. The consistent pattern observed in these mice was the partial formation of the cuticle on some fibers with absence on other fibers, which led Luong et al. to suggest that the inner root sheath may not be normal. *cux-1ΔC/ΔHD* mutant mice did not possess vibrissae in their muzzle skin. Instead, only lance-shaped broken ends of hair fibers and irregularly wavy hairs were observed that were short and similar to the coat hair. Follicular dystrophy and progressive alopecia was observed in *cux-1ΔC/ΔHD* homozygotes as they aged. In some instances dystrophic fibers pushed out of growing follicles, twisted below the level of the sebaceous gland, and entered the dermis and hypodermis, with an inflammatory response being found in and around the fiber. Therefore, the *cux-1ΔC/ΔHD* mutation apparently causes abnormal formation of the coat and vibrissa hair fibers similar to that of the *cux-1ΔCR1* mutation.

The function of Cux-1ΔC/ΔHD is different compared to wild-type Cux-1 protein. Immunofluorescence revealed that expression of the *cux-1ΔC/ΔHD* mutant protein is restricted to the cytoplasm instead of the nucleus where full length Cux-1 is normally expressed (38,39). The C-terminus of Cux-1 or the HD may be required for Cux-1 nuclear localization. It is feasible that these regions of Cux-1 may contain critical nuclear localization signals (NLS) that play a major role in the localization of Cux-1 to the nucleus. Luong et al.(38) suggested that since Cux-1 expression within

the nucleus is absent, then Cux-1 protein function may also be absent. This result was supported by the observation that DNA binding activity of HiNF-D was lost in nuclear extracts derived from mouse embryonic fibroblasts (MEFs) or adult tissues of homozygous mutant *cux-1ΔC/ΔHD* mice (38,39). They concluded that deletion of the C-terminus containing HD results in a molecular phenotype reflected by the absence of Cux-1 containing protein/DNA complexes. Histone H4 mRNA levels from wild-type, heterozygous, and homozygous mutant MEFs were similar, indicating that there is no difference in the amount of cells present in S phase. This was supported by the observation that *cux-1ΔC/ΔHD* MEFs had a growth pattern that was similar to wild type MEFS(38, 39). The effect of *cux-1ΔC/ΔHD* mutation may be dependent on the specific cell-type.

In contrast to *cux-1ΔC/ΔHD* mice (38,39), CUTL1^{laCZ} mice (40) lack CR3 and HD in addition to the C-tail being deleted. Mutant mice on inbred genetic backgrounds are born at a mendelian frequency. Similar to *cux-1ΔC/ΔHD* mice, CUTL1^{laCZ} die shortly after birth because of delayed differentiation of lung epithelia. The CUTL1^{laCZ} gene is crossed onto an outbred genetic background, there is a less pronounced delay in lung development which permits CUTL1^{laCZ} mice to survive beyond birth. Another similarity between *cux-1ΔC/ΔHD* and CUTL1^{laCZ} is the overall growth inhibition runt phenotype as well as disruption of hair follicle morphogenesis resulting in an abnormal pelage(38-40). CUTL1^{laCZ} mice have a reduced inner root sheath (IRS), and the transcription of Sonic hedgehog and IRS-specific genes were found to be deregulated in CUTL1^{laCZ} mutant hair whisker

follicles. This result is consistent with the specific expression of CUTL1^{laCZ} in the progenitors and cell lineages of the IRS. Similar to Cux-1ΔC/ΔHD protein, the Cux-1-LACZ fusion protein expression was restricted to the cytoplasm and failed to repress gene expression (38-40). Both CUTL1^{laCZ} and *cux-1ΔC/ΔHD* mutations are deletions of the C-tail encompassing the HD. The difference between CUTL1^{laCZ} and Cux-1ΔC/ΔHD is the absence of the CR3 domain. Since CUTL1^{laCZ} and Cux-1ΔC/ΔHD have similar phenotypes the loss of the CR3 domain has no effect on altering the phenotype observed when HD and the C-tail are removed. Therefore, CR3 may play a very mild role in Cux-1 function. In all three Cux-1 mutant models (*cux-1ΔCR1*, CUTL1^{laCZ} , *cux-1ΔC/ΔHD*) a defect in hair and follicle development was observed. Loss of CR1 resulted in a mild hair and follicle defect. In contrast, loss of CR3, HD and the C-tail of Cux-1 resulted in more severe defects. The loss of HD and the C-terminus results in a mutant protein that does not localize to the nucleus and can not repress promoter activity. Since loss of CR1, the Cux-1ΔCR1 protein, results in what appears to be a more stable protein-DNA interaction then wild-type Cux-1, these results collectively support *in vivo* observations that CR1 may function to inhibit stable DNA binding and that CR3 and HD domains are required for stable DNA binding interactions (32).

Since Cux-1 is known to be highly expressed in proliferating cells it became a point of interest to determine how overexpression of Cux-1 might alter mouse development. Therefore, our laboratory designed transgenic mice whereby expression of Cux-1 was driven by the CMV promoter (CMV/Cux-1 Transgenic)

(20). The overexpression of Cux-1 resulted in transgenic mice with multiorgan hyperplasia and organomegaly (**Figure 1-4**), but not an overall increase in body size. Organomegaly was observed in heart, kidney testis, spleen, seminal vesicle and liver. Cux-1 Transgenic mice had Glomerular abnormalties which included the replacement of normal squamous epithelium of Bowman's capsule with proximal tubule-like epithelial cells (20). These cells were continuous with the proximal tubule epithelium and were observed in all glomerulae. We found that increased expression of Cux-1 in mesangial cells results in cell proliferation and mesangial expansion. In addition, these changes are potentially related to disruption of podocyte architecture leading to loss of filtration. These results suggest that expression of Cux-1 is sufficient to induce the early events of mesangioproliferative glomerulonephritis.

Transgenic kidneys also exhibited glomerular and tubular hypercellularity (20,42). CMV/Cux-1 Transgenic kidneys had a significantly increased renal cortex, without an overall increase in nephron number. However, there was an overall increase in the total number of cells in both the cortex and glomerulae which correlated with an increase in cell proliferation within these respective regions. Further analysis revealed that p27 mRNA and protein expression levels were downregulated in CMV/Cux-1 transgenic mice. Since the CMV/Cux-1 Transgenic mice had a similar phenotype to that of the p27 (-/-) mice our laboratory has focused on investigating the function of Cux-1 to regulate the cell-cycle. We found that increased levels of Cux-1 transfected into HEK293T cells, results in the reduction of

p27 promoter luciferase activity implicating p27 as a target gene for Cux-1 repression. We found that liver hepatomegaly within CMV/Cux-1 Transgenic mice correlated with an increase in cell proliferation (43). In addition, the increase in Cux-1 expression in transgenic livers was associated with a decrease in p21, but not p27, expression. Within transgenic livers, Cux-1 was ectopically expressed in a population of small cells, but not in mature hepatocytes, and many of these small cells expressed markers of proliferation (43). Transgenic livers showed an increase in alpha-smooth muscle actin, indicating activation of hepatic stellate cells, and an increase in cells expressing chromogranin-A, a marker for hepatocyte precursor cells. Morphological analysis of transgenic livers revealed inflammation, hepatocyte swelling, mixed cell foci, and biliary cell hyperplasia (43). These results suggest that increased expression of Cux-1 may play a role in the activation of hepatic stem cells, possibly through the repression of the cyclin kinase inhibitor p21.

The full length Cux-1 protein can be proteolytically processed by Cathepsin-L into a p110, p90 or p75 protein (32, 33). The p75 protein isoform was found to be overexpressed in human breast cancers (41). Cadieux et al.(41) designed transgenic mice that overexpress p75 driven by the mouse mammary tumor virus-long terminal repeat. They found that 33% of mice from two transgenic lines and from backcrosses developed a similar disease characterized by splenomegaly, hepatomegaly, and frequent infiltration of leukocytes into nonhematopoietic organs such as the kidneys and lungs. They also found an increase in the overall number of neutrophils in the blood, spleen, and bone marrow which correlated with anemia and

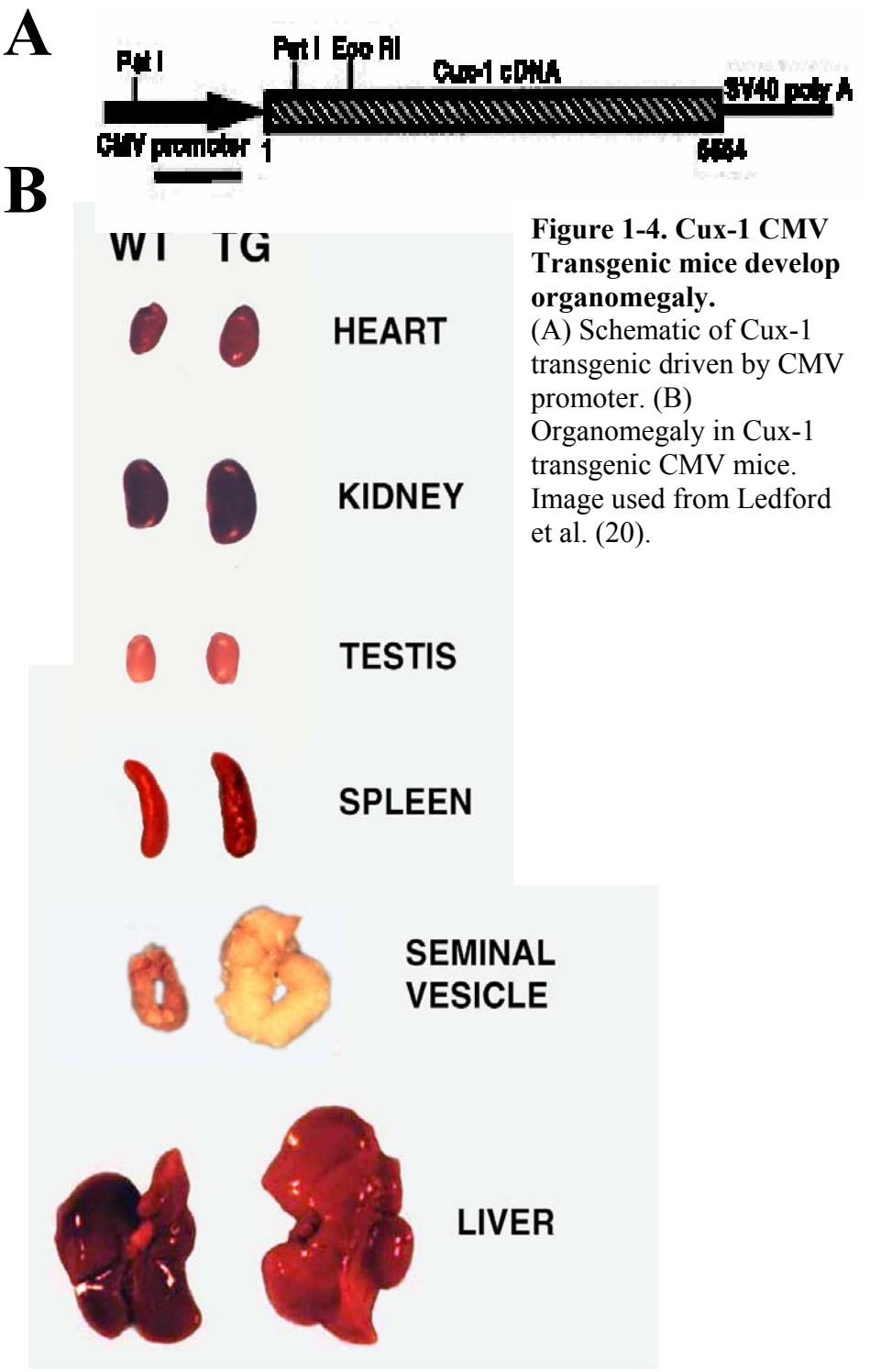


Figure 1-4. Cux-1 CMV Transgenic mice develop organomegaly. (A) Schematic of Cux-1 transgenic driven by CMV promoter. (B) Organomegaly in Cux-1 transgenic CMV mice. Image used from Ledford et al. (20).

thrombocytopenia. Cadieux et al.(41) therefore concluded that Cux-1 p75 transgenic mice were susceptible to myeloproliferative disease-like myeloid leukemia and that overexpression of p75 might disturb hematopoietic homeostasis in some way.

It is very interesting that the phenotype of spleenomegaly and hepatomegaly are observed in both CMV/Cux-1 Transgenic (20) and Cux-1 p75 transgenic mice (41). The phenotype observed in CMV/Cux-1 Transgenic might results from both the full length Cux-1 p200 protein and Cux-1 p75. It is possible that overexpression of Cux-1 in the CMV/Cux-1 transgenic mice results in higher then normal Cux-1 p75 levels. To date, we have never analyzed p75 expression in CMV/Cux-1 Transgenic mice. It is also conceivable that Cux-1 p110 and Cux-1 p90 also may be abnormally expressed to help produce the CMV/Cux-1 transgenic phenotype.

The overexpression of Cux-1 p200 and the production of Cux-1 p110, Cux-1 p90, Cux-1 p75 may be a tissue-specific event. The observation that Cux-1 p75 transgenic mice may have a myeloproliferative disease (41) is consistent with the function of Cux-1 to regulate the immune system in that hyperplasia of myeloid cell types and reduced thymic cellularity were observed in *cux-1*Δ*C/*Δ*HD* mice. The mechanism for Cux-1 regulation of immunological processes still remains unclear.

There has been a significant amount of information extracted from using mouse models to study the function of the murine homeobox gene Cux-1. It is still not clear how the individual loss of CR2, CR3, and HD with the retention of the full length Cux-1 sequence, might affect mouse development. Mice lacking both CR1 and CR2 domains, CR2 and CR3 domains, and CR3 and HD domains may provide

additional insight into Cux-1 function. Furthermore, the cleaved portion of Cux-1, Cux-1p110 has not been evaluated in animal models. A transgenic mouse that overexpresses Cux-1 p110 may be very useful in defining the role of Cux-1 to act as a transcription factor during S-phase of the cell cycle. The role of Cux-1 p110 in participating in the progression of cells through the cell cycle towards cell division has not been evaluated *in vivo* when Cux-1 p110 is overexpressed. The advent of Cre-Lox tissue specific expression systems may also be utilized to determine how tissue specific expression of Cux-1 may affect, hair development, organogenesis and sexual reproduction. The current wealth of knowledge of the *in vivo* function of Cux-1 can only be overshadowed by the next accumulation of knowledge gathered in future studies.

Table 1-2. Genes that are targets for Cux-1 repression.
Modified from Nepvue (13).

Genes	References
γ-globin (human)	Mantovani et al. 1989; Ottolemghi et al, 1989
β-globin (Xenopus laevis)	Patient et al. 1989
gp91-phox	Skalnik et al, 1991; Neufeld et al, 1992; Lievens et al. 1995
γ-globulin	Superti-Furga et al., 1989
β-MHC gene	Andres et al. 1992
NCAM (neural cell adhesion molecules) gene	Valarche et al. 1993
Tyrosine hydroxylase	Yoon and Chikaraishi, 1994
CD8a gene, matrix attachment region (MAR)	Banan et al. 1997
Human Papillomavirus Type 6 Long Control Region	Pattison et al., 1997; Ai et al., 1999; O'Connor et al., 2000
Neutrophil collagenase	Lawson et al., 1998
p21 Waf/Cip/Sdi1	Coqueret et al., 1998
T cell receptor β gene (MAR)	Chattopadhyay et al. 1998
Cystic fibrosis transmembrane conductance regulator gene	Li et al., 1999
Osteocalcin gene	Van Gurp et al., 1999
TGFβ Type II Receptor	Jackson et al., 1999
Immunoglobulin heavy chain enhancer	Wang et al., 1999
Tryptophan hydroxylase	Teerawatanasuk et al., 1999
p27 (kip1)	Ledford et al., 2002
CXCL1	Nirodi et al., 2002
PCNA (Drosophila)	Seto et al., 2006

Cux-1 function

Cux-1 DNA binding activity was initially characterized as CCAAT displacement activity (44) or participating in complex with histone nuclear factor D (HiNF-D) (45) with binding activity disappearing when myeloid cells differentiated. Cux-1 binding activity was downregulated during fetal liver development (45) and osteoblast differentiation (46,47). **Table 1-2** shows a list of all genes that are targets for Cux-1 mediated repression. Many of the targets of Cux-1 are genes that are repressed in proliferating precursor cells and are turned on as cells become terminally differentiated as Cux-1 activity ceases. Cux-1 homologues predominately function as transcriptional repressors of many different genes (28, 44-49).

Figure 1-3 shows that Cux-1 contains four DNA binding domains (CR1, CR2, CR3, and HD). While CDP/Cux p200 only transiently binds to DNA and carries the CCAAT-displacement activity, p110 makes a stable interaction with DNA (27,43). Moon et al. (50, 51) demonstrated that full length Cux-1 protein, containing all four DNA binding domains, displays rapid and transient DNA binding activity. The NH_2-terminal end of the full-length protein harbors an autoinhibitory domain that inhibits DNA binding (52). *In vitro* studies using CR fusion proteins demonstrated that Cux-1 domains (CR1CR2, CR3HD, CR1HD, CR2HD and CR2CR3HD) bind DNA with variable affinity (51). The CR2CR3 fusion protein did not efficiently bind to DNA. The CR1CR2 domains were found to bind a GC rich sequence (GC box) of DNA transiently and rapidly. CR1 CR2 bound to two CA/GAT sites, organized as

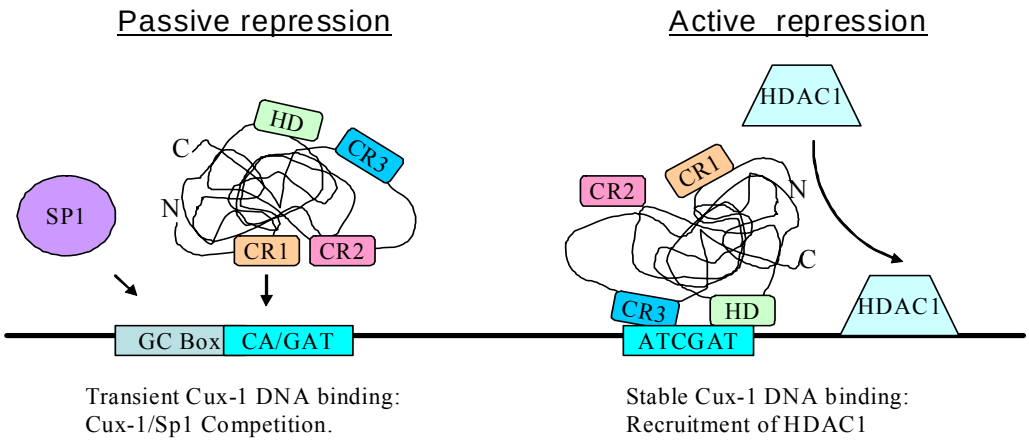


Figure 1-5. Model for DNA binding domains of Cux-1 mediating transcriptional repression. Passive repression involves CR1 and CR2 competing with SP1 for binding to a promoter. Active repression involves CR3 and HD stably binding to DNA and recruiting proteins such as HDAC1 in mediating repression of gene transcription.

direct or inverted repeats. The Sp1 transcriptional activator also binds the GC box (53). Based primarily from work done *in vitro*, a model for Cux-1 DNA binding was proposed (13, 48, 54) based on the difference in Cux-1 DNA binding domain properties (**Figure 1-5**). CR1 and CR2 are thought to compete with Sp1 for occupancy of a GC box. The binding of CR1CR2 is considered "passive repression" because transcription is repressed indirectly by Cux-1 out-competing Sp1 for GC box binding. The presence of Cux-1 on the GC box instead of Sp1 would prevent Sp1 from activating transcription. Since results from our lab demonstrated that ectopic expression of Cux-1 in CMV/Cux-1 Transgenic mice leads to the downregulation of p27 mRNA and protein levels (20), we predicted that Cux-1 would similarly bind to the p27 promoter. Recently, by performing Chromatin Immunoprecipitation analysis (ChIP) we found that Cux-1 binds to a region of the cyclin kinase inhibitor p27

promoter that contains a GC box in the vicinity of a CCAAT box (Unpublished observation). It has also been demonstrated that Cux-1 binds the promoter of the cyclin kinase inhibitor p21 to a GC box (55). Given that Cux-1 binds to the GC box of the promoter where Cux-1 is found to bind, it is very likely that Cux-1 binds transiently to p21 and p27 promoters and may passively repress gene expression by competing with Sp1 for promoter occupancy.

The *in vitro* examination of independent Cux-1 binding domains determined that CR3HD stably bind DNA (54). The product of stable Cux-1/DNA interaction is the ability of Cux-1 to possibly recruit histone deacytlases (HDAC) such as HDAC1 (56). Two active transcriptional repression domains present within the C-terminus of Cux-1 appears to associate with HDACs (48, 56). Two closely linked transcriptional repression domains are located within the distal C-terminal of Cux-1. Mailly et al. suggested that the ability of Cux-1 to stably bind DNA and exhibit "active repression" involves the Cut homeodomain and the C-terminus of Cux-1 since HDAC1 can be pulled down by a GST-CDP/Cut fusion protein containing the homeodomain and C-terminus (48, 56). HDAC1 could then deacetylate chromatin to repress gene expression. The physical occupancy of Cux-1 at a promoter may provide a physical barrier to prevent transcriptional activators and the normal transcriptional machinery (i.e RNA Polymerase) from interacting with the promoter. Goulet et al. (32) demonstrated that the full length Cux-1 protein, termed P200, transiently binds to DNA. They found that a nuclear form of Cathepsin-L, a proteolytic enzyme that normally localizes to the lysosome, cleaves the Cux-1 P200

into a p75 and p110 forms. More recently, it has been demonstrated that Cathepsin-L can also cleave Cux-1 into a p90 protein (33). In addition, a tissue-specific mRNA species was found to code for the p75 CDP/Cux isoform which contains only two DNA-binding domains: CR3 and HD (32, 41). Molecular studies showed that the full-length protein, p200, binds rapidly but transiently to DNA and carries the CCAAT displacement activity (50, 51). In contrast, the p110 and p75 isoforms behave like classical transcription factors that engage in slow but stable interactions with DNA (59). The p110 protein may participate in transcriptional activation and can stimulate cell proliferation by accelerating entry into S phase (57,58). It has been proposed that the N-terminus of Cux-1 contains an inhibitory DNA binding domain. (52) Goulet et al. (32) suggested that the CR1 domain is an autoinhibitory domain which could prevent stable Cux-1 binding. They reasoned that the full length P200 protein might bind to DNA in a transient fashion because it contained the CR1 domain which might inhibit stable DNA binding promoted by the CR3 and HD. The p110 protein lacks the N-terminus and the CR1, but retains CR2, CR3 and HD and has been demonstrated to have a very stable interaction with DNA (32). It is possible that CR1 and the N-terminus mask the CR3 and HD resulting in transient Cux-1 DNA binding. The removal of the N-terminus could then "activate" Cux-1 to bind to DNA by allowing CR3 and HD to freely bind to DNA resulting in a more stable Cux-1– DNA interaction. Unfortunately, the Cux-1 protein has not been studied by X-ray Crystallography to date so the three-dimensional conformation of Cux-1 is currently not known.

Work from animal model studies has revealed important characteristics of Cux-1 DNA binding activity. Cux-1 protein lacking the C-tail, containing either the HD or both the CR3 and the HD, has reduced DNA binding activity (38, 40). Expression of both of these proteins is restricted to the cytoplasm, suggesting that a nuclear localization sequence (NLS) may be present within the C- tail. It should not be discounted that the CR3 and HD domains are important for Cux-1 localization within the nucleus. Hypomorph deletions of CR3 and HD while retaining the C-tail have not as of yet been generated. Studies where the C-tail of Cux-1 is deleted in mice highlight the importance of both CR3 and HD domains for Cux-1 DNA binding as mutant Cux-1 exhibited limited DNA binding. Tufarelli et al.(37) showed that the Cux-1ΔCR1 protein is less readily competed off with cold radio-labeled oligonucleotide then wild-type Cux-1 protein in electromobility-shift assay competition studies. However, Cux-1ΔCR1 protein expression levels appeared to be higher then wild-type Cux-1 in both Spleen and Thymus and thus the observation that Cux-1ΔCR1 stably binds to DNA might be a concentration dependent result of increased Cux-1ΔCR1 expression. Based upon the abundant evidence that Cux-1 gene targets are repressed in cells containing high levels of Cux-1 (**Table 1-2**), the activity of Cux-1 mediated repression may be completely concentration dependent. Transition between the inactive and active states of Cux-1 for DNA binding *in vivo* appears to be governed by post-translational modifications and/or interactions with one or more protein partners (62-71). Cux-1 may function as a transcriptional activator or repressor depending on the cell–type in which it is expressed.

The function of Cux-1 as a transcription factor might depend upon cell-type and expression of proteins (29, 66) within a cell at specific stages of the cell-cycle. Results in mammals (20, 37-43) suggest that Cux-1 expression or activity is restricted to proliferating cells establishing an important role for Cux-1 in developmental organogenesis of such organs as the kidney (19, 20), lung (38-40) and liver (20, 43). The nature of how Cux-1 p110, p90 and p75 interact with nuclear proteins and form discrete protein complexes involved in transcriptional regulation is still not clear. Much of the studies to date have involved analysis of the full length Cux-1 p200 and Cux-1 p110 in regard to protein-protein interactions as well as DNA binding interactions. Studying truncated Cux-1 protein interactions during late G1 and early S-phase may elucidate the function of Cux-1 to regulate the cell-cycle.

Role of Cux-1 in cell-cycle regulation

Regulation of the cell-cycle is critical to the life of an organism. The cell-cycle is tightly regulated to ensure proper replication of select cells (**Figure 1-6**). Inappropriate progression through the cell-cycle may lead to aberrant cellular proliferation. The consequences of abnormal cell division can be catastrophic to an organism and may result in mortality. Therefore cells are required to pass through two "checkpoints" before being permitted to divide. The first checkpoint is between G1 and S-phase transition while the second is between G2 and M-phase. The G1-S checkpoint serves to prevent cells that incurred DNA damage from progressing through the cell cycle. Missense, nonsense and deletion mutations in critical genes

may damage a parental cell. The G1-S checkpoint restricts cell-cycle progression to cells with undamaged or repaired DNA (via base excision repair, homologous recombination repair etc.). Moreover, the G1-S checkpoint ensures viable future generations of cells by not producing cells with lethal inheritable mutations.

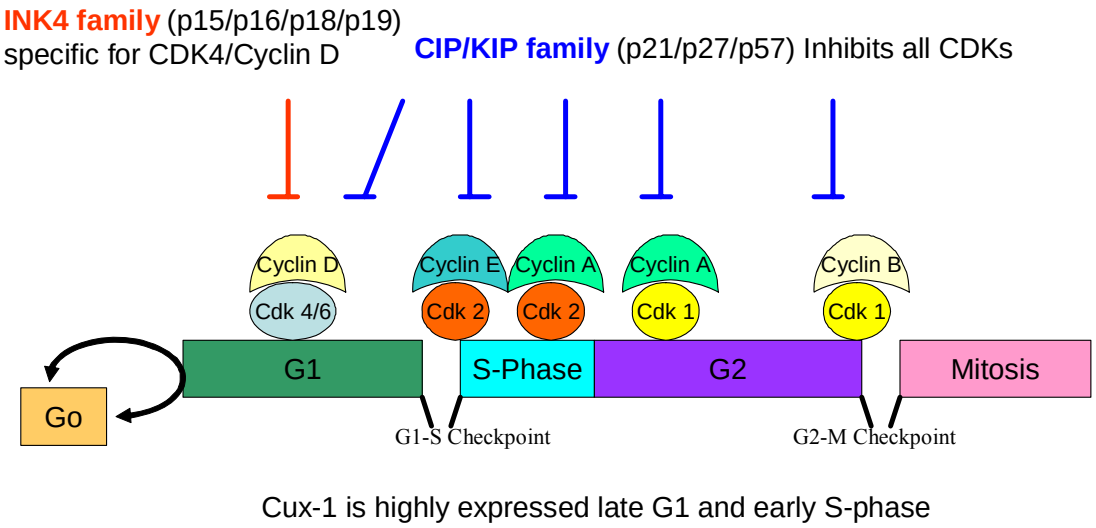


Figure 1-6. Cell Cycle regulation. The cell cycle is tightly regulated by positive factors (cyclin-CDK complexes) which favor progression through the cycle and negative factors (cyclin kinase inhbitors- 2 families: INK4 or CIP/KIP) which restrict progression through the cell cycle.

Cell cycle is regulated by nuclear proteins that can positively (cyclins and cyclin dependent complexes) or negatively (cyclin kinase inhibitors) control cell proliferation. Cyclins become upregulated during the cell cycle and form complexes with cyclin dependent kinases (CDK) to specifically regulate genes involved in each successive phase of the cycle (**Figure 1-6**). During late G1 and early S-phase CDK 4/6-cyclin D complexes phosphorylate the protein retinoblastoma (RB) releasing its

hold on the transcription factor E2F which activates genes involved in DNA synthesis (**Figure 1-7**). In contrast, cyclin kinase inhibitors (CKIs) are nuclear proteins that bind to CDK-cyclin complexes to prevent the phosphorylation of RB. CKIs therefore prevent E2F release and gene regulation of components necessary for DNA synthesis resulting in cell cycle arrest and attenuated cell proliferation.

There are two classes of CKIs, the CIP/KIP and ink4 family. The CKIs p21 and p27 regulate progression through the cell cycle at the G1-S transition. Both p21 and p27 contain an N-terminal CDK-binding domain. (75,76) This domain allows p21 and p27 to bind to CDK2/4 and inhibit the formation of CDK2/4- cyclin D1 complex. This leads to inhibition of CDK2/4-cyclin D1 kinase activity which prevents the activation of transcription factors required for S-phase. Gene Targets of Cux-1 mediated repression are repressed in proliferating precursor cells (See **Table 1-2**) and are turned on as cells become terminally differentiated and Cux-1 activity declines (27). A role for Cux-1 in cell-cycle progression was suggested from the findings that Cux-1 DNA binding activity oscillates during the cell cycle, reaching a maximum at the end of G1 and during the S Phase. (17) A recent model has been proposed (27,30,31,51) whereby full length Cux-1 p200 is cleaved by Cathepsin-L, during S-phase of the cell cycle, into an N-terminally truncated p110 protein (**Figure 1-8**). Cux-1 p200 has also been demonstrated to be cleaved into a p90 protein and a p75 protein.(49,30) The p110 protein exhibited stable DNA binding interactions.(30) Coqueret et al.(17) demonstrated that Cux-1 binds to the p21 cyclin kinase inhibitor promoter and represses p21 expression. Overexpression

of Cux-1 in CMV/Cux-1 transgenic mice results in the down regulation of the p27 cyclin kinase inhibitor (20). When full length Cux-1 was overexpressed in increasing concentrations with a reporter p27 luciferase promoter, reduction of promoter activity was observed (20). Since Cux-1 is highly expressed at the end of G1 and during early S phase (51) and can repress both p21 and p27 promoter activity (20, 55), Cux-1 appears to function to permit progression through the cell-cycle leading towards cell division through the repression of the cyclin kinase inhibitors p21 and p27.

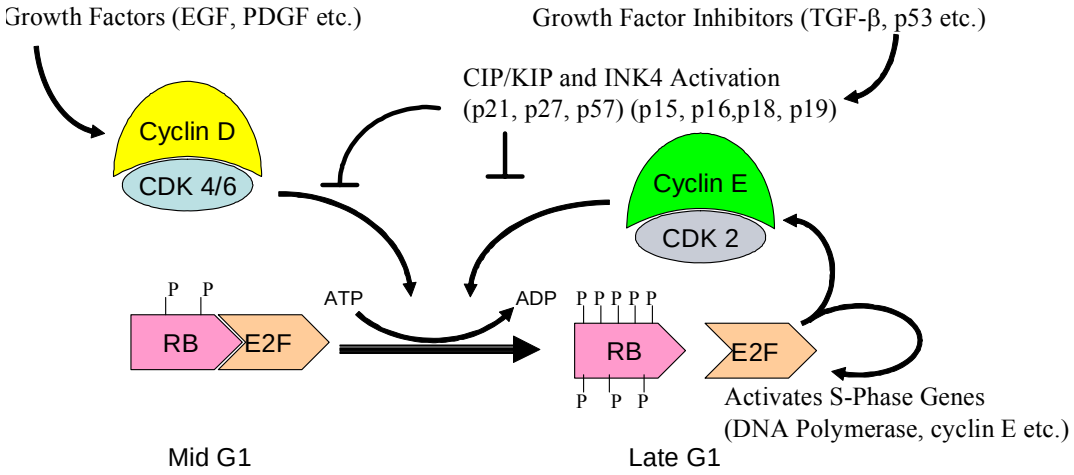


Figure 1-7. Regulation of the G1-S checkpoint of the cell-cycle. The RB protein sequesters E2F transcription factor during mid G1. Growth factors such as EGF stimulate CDK 4/6. This complex phosphorylates RB removing its ability to bind E2F during late G1. E2F then activates genes required for progression into S-phase. Growth factor inhibitors rush as TGF-β can activate cyclin kinase inhibitors capable of preventing CDK-cyclins from phosphorylating RB.

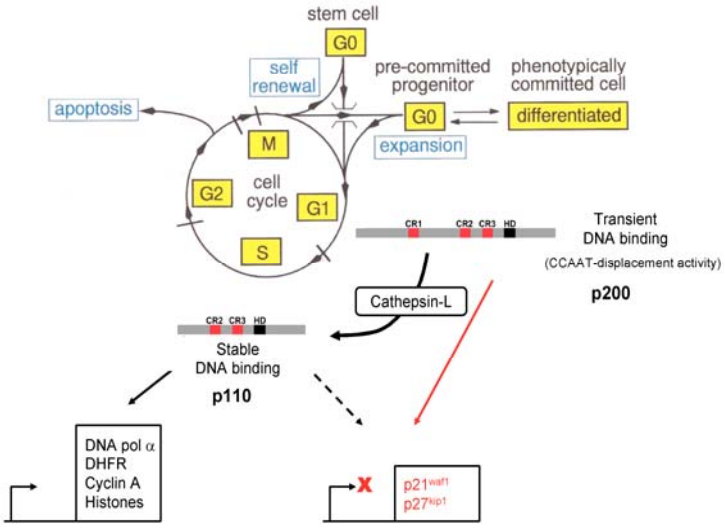


Figure 1-8. Model for Cathepsin-L mediated degradation pathway of Cux-1 initiated during S-phase. Full length Cux-1 (p200) is proteolytically cleaved to Cux-1 (p110 protein) during S-phase by Cathepsin-L. This might be the initial step required for Cux-1 proteolysis. Cux-1 (p110) protein stably binds DNA while Cux-1 (p200) binds transiently to DNA.

Cux-1 is the DNA binding subunit of the HinF-D complex that forms during S-phase (69, 72,73). The HinF-D complex consists of Cux-1, CDK2/CyclinA and pRB/p107 (69). The HinF-D complex is involved in the activation of histones during S-phase (72,73). The HinF-D mediated pathway is a mechanism of gene regulation at G1/S that is independent of E2F (68). Cux-1 DNA binding activity is highest during early S-phase and gradually declines as S-phase is completed (51). It is possible that depending upon the cell type, there may be lower or higher amounts of Cux-1 p200, Cux-1 p110, Cux-1 p90, and/or Cux-1 p75 during S-phase. Moreover, the protein binding partners that various truncated forms of Cux-1 may interact with during S-phase is unclear. It is possible that the Cathepsin-L cleavage products of Cux-1 undergo many unique post-translational modifications during S

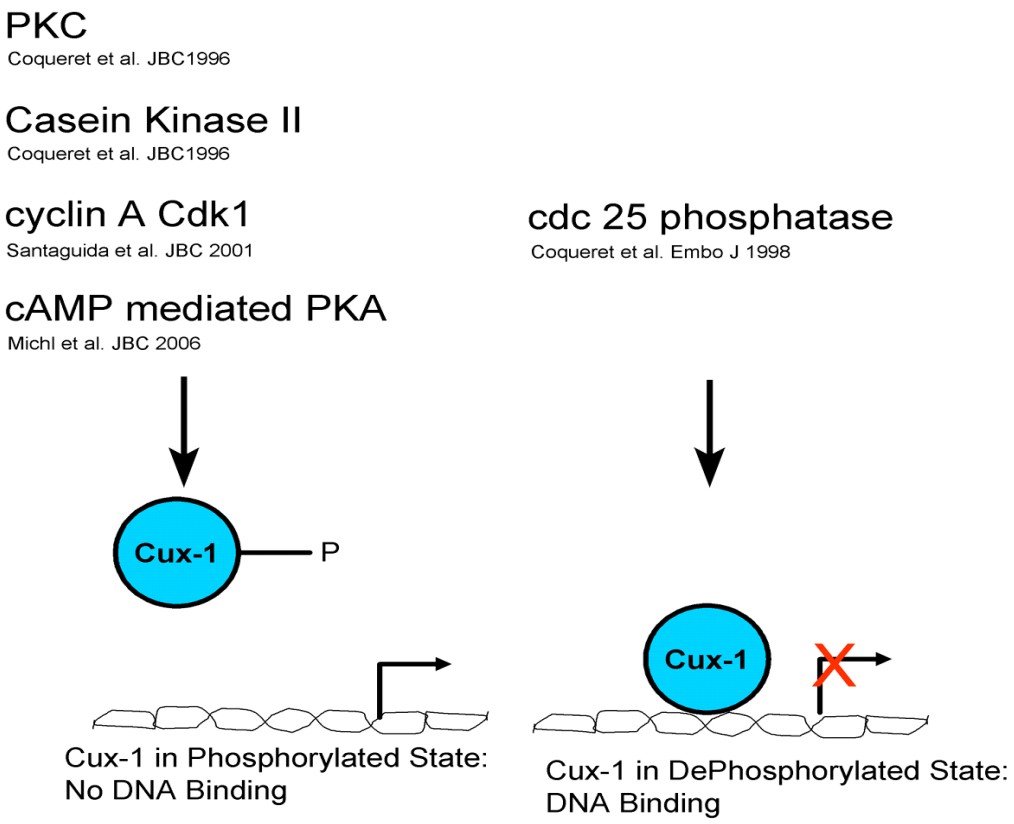


Figure 1-9. Cux-1 DNA binding activity is regulated by phosphorylation/dephosphorylation mechanism. PKC, Casein Kinase II, cyclin A CDK1 and PKA phosphorylate Cux-1 resulting in decreased Cux-1 binding to DNA. In contrast, when Cux-1 is dephosphorylated by cdc phosphatase Cux-1 binds to DNA.

phase which could determine Cux-1 DNA binding ability directly and involvement in complex protein-protein interactions.

When Cux-1 is phosphorylated Cux-1 DNA binding is inhibited (55, 59, 60). In contrast, when Cux-1 is dephosphorylated Cux-1 DNA binding is permitted (**Figure 1-9**). Coqueret et al. (55) found that cdc25A dephosphorylates Cux-1 which would permit Cux-1 DNA binding during S-phase. At the beginning of S-phase Cux-1 p200 is cleaved to Cux-1 p110 (32). Santaguida and Nepveu (73) found that

Cyclin A/CDK2 does not phosphorylate Cux-1 p110. Moreover, they determined that Cyclin A/CDK2, is expressed during S-phase of the cell-cycle, did not inhibit Cux-1 DNA binding activity and transcriptional activity. However, Cyclin A/CDK1, which is highly expressed during G2 of the cell-cycle, phosphorylated Cux-1 p110 protein resulting in the inhibition of DNA binding and transcriptional activity (73). These results may help to explain why Cux-1 p110 DNA binding activity is maximal during S-phase and decreases in G2.

Cux-1 clearly plays a role in regulating the cell-cycle by repression of p21 and p27 cyclin kinase inhibitors. However, the exact mechanism is unclear. It is possible that Cux-1 forms various complexes with different proteins and/or may act as an independent factor depending on cell type. Nishio et al. (75) reported that Cux-1 interacts with G9a Histone Lysine Methyltransferase (HKMT) at the site of the p21 promoter. HKMT can methylate Histone 3 lys-9 within the Cux-1 regulatory site of the p21 promoter. Methylation of the amino acid lysine results in gene-silencing. Therefore this is one possible manner by which Cux-1 can mediate repression of p21 expression. We found that Cux-1 interacts with TLE-4, (70) the mammalian homologue of the drosophila gene Groucho, in rat kidney epithelial cells that consistituitively express Notch correlated with downregulation of p27. However, evidence of Cux-1 and TLE-4 interaction as a requirement for regulation of p27 promoter activity is still not clear. The pRB protein has been suggested to function as a co-repressor when bound to Cux-1 as Cux-1 pRB interactions were required for repression of Histone 4 (H4) expression (79). Cux-1 has been reported to be a

member of different protein complexes. Cux-1 is part of the NF-mUNR complex to repress immunoglobulin (77). It is a complex consisting of the histone acetyltransferase p300, CBPAF and CBP (63). Cux-1 may play a role as a structural component of chromatin through its interaction with nucleosomal DNA and association with nuclear matrix attachment regions (13). The role of Cux-1 in the nucleus appears to depend on the stage at which a cell is in within the cell-cycle. Cux-1 is highly expressed during late G1 and early S-phase.

Regulation of Cux-1 by post-translational modification

Numerous transcription factors such as nuclear factor kappaB undergo an "activation/inhibition-degradation" pathway during critical stages of the Cell-Cycle (13, 78-86). Proteolytic cleavage, phosporylation/dephosphorylation, acetylation and ubiquitization pathways serve as post-translational modification mechanisms to activate or inhibit protein activity during time-points that are appropriate for proper cellular function. There are atleast three ways by which Cux-1 can undergo post-translational modification; Proteolytic cleavage/processing (32, 33, 51), phosphorylation/dephosphorylation (59-61) and Acetylation (63). The post-translational modification of Cux-1 seems to play an important role in the function of Cux-1. Goulet et al.(32) found that a nuclear form of Cathepsin-L is present during S-Phase of the cell-cycle and can proteolytically cleave full length Cux-1 (p200) to a p110 and p75 protein. The p75 protein undergoes rapid degradation while the P110 protein was found to stably bind to DNA. The cleavage of Cux-1 by Cathepsin-L

appears to regulate the ability of Cux-1 to bind to DNA and act as a transcription factor. Therefore, the regulation of nuclear Cathepsin-L may serve as a determinate factor of Cux-1 function. Weak Cux-1/ DNA binding was observed in G0 and early G1, unless cell extracts were previously treated with alkaline phosphatase. In contrast, strong Cux-1/DNA binding was observed in S-phase and was shown to result both from an increase in Cux-1 expression and dephosphorylation by the Cdc25A phosphatase.

Cux-1 function appears to be regulated by phosphorylation and dephosphorylation (Figure 9). Phosphorylation of Cux-1 by Casein Kinase II (59) and PKC (60) has been demonstrated *in vitro* and *in vivo*. The phosphorylation of Cux-1 restricts Cux-1 DNA binding ability whereas dephosphorylation by cdc25A phosphatase permits Cux-1 DNA binding ability (55). PKC phosphorylates Cux-1 on Threonine 415 within CR1, Threonine 804 within CR2 and Serine 987 of CR3 (60). cAMP mediated PKA has also been demonstrated to phosphorylate Cux-1 (61). Casein Kinase II phosphorylates Cux-1 on Serine 400 of CR1, Serine 789 of CR2 and Serine 972 of CR3 (59). Calcineurin is a Serine/Threonine phosphatase. In addition to phosphorylation of Cux-1 resulting in the inhibition of Cux-1 binding to DNA, it was also determined that acetylation also inhibits Cux-1 DNA binding. PCAF-mediated acetylation of the homeodomain of Cux-1 was shown to inhibit DNA binding (63).

Signal transduction pathways involving Cux-1

In Drosophila, multiple genetic interactions between Cut and the Notch and Wingless signaling pathways occur during wing development. To begin to determine whether Cux-1 regulation by the Notch signaling pathway is conserved in mammals, we compared the expression patterns of Cux-1, the murine Notch receptors (Notch 1-4), and the murine ligands (Jagged 1, Jagged 2, and Delta 1) during murine embryogenesis and kidney development (70). We demonstrated that Cux-1 expression overlaps with that of Notch signaling pathway components in multiple tissues during embryonic development. Furthermore, Cux-1 was significantly up-regulated in the rat kidney epithelial cell line RKE expressing a constitutively active Notch 1, and this finding was associated with a reduction of p27. Moreover, Cux-1 interacts with the Groucho homolog TLE-4, a co-repressor recruited by Notch effector proteins. Taken together, these results suggest that Cux-1 may function in the context of the Notch signaling pathway in multiple tissues during mammalian embryogenesis. Cathepsin-L, cAMP mediated PKA, Casein Kinase II, PKC, PCAF, Cyclin A CDK1, cdc25 phosphatase all have direct effects on the function of Cux-1 to bind to DNA. These signaling factors are downstream mediators of potentially many upstream signaling pathways within a cell that could involve cross-talk between signaling pathways.

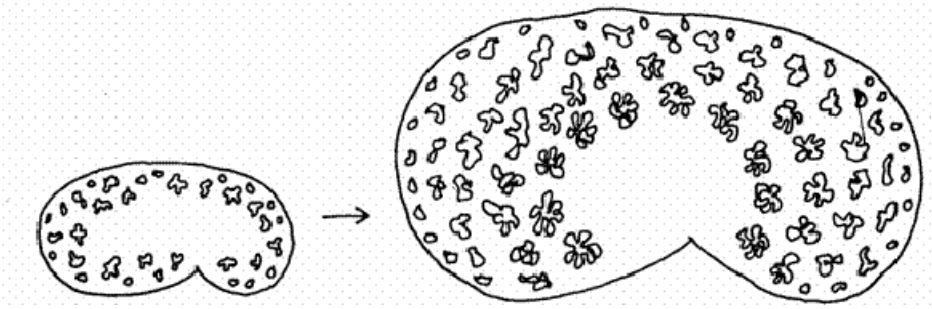


Figure 1-10. New nephrons develop in a centrifugal pattern. Developing Nephrons begin to grow along the outer edge of the kidney in an area termed the nephrogenic zone. Mature nephrons can be found within the interior region of developing kidneys.

Role of Cux-1 in kidney development

In Drosophila, malpighian tubules are the insect excretory organs that serve as primitive kidneys. Deletions of the *cux-1* gene prevent formation of malpighian tubules indicating that *cux-1* is required for normal development (8). In mammals, renal development proceeds through three stage specific structures: the pronephros, the mesonephros, and the metanephros (Reviewed in 87-89). The first two stages are transient and involute *in utero*, while the remaining metanephros develops into the definitive kidney. The kidney develops by a reciprocal induction mechanism (**Figure 1-10**).

Development is originated by the metanephric mesenchyme triggering ureteric bud projection from the wolffian duct into the metanephric mesencyhme. The induction of the metanephros can be viewed as a dialogue between the ureteric bud and the metanephric mesenchyme. GDNF is synthesized and secreted by the

metenphric mesenchyme and binds tyrosine kinase RET receptors present on the wolffian duct and ureteric buds. GDNF receptor binding on the wolffian duct induces initial ureteric budding into the metanephric mesenchyme as well as secondary ureteric bud branching once the ureteric bud has entered the mesenchyme. The tips of these branches induce the loose mesenchymal cells to form epithelial aggregates. Each aggregated nodule of cells will proliferate and differentiate into a unique structure of a renal nephron. Each condensate first elongates into a "comma" shape, and then forms a characteristic S-shaped tube. Soon afterwards, the cells begin to differentiate into regionally specific cell types that include distal and proximal tubule cells. The ureteric bud then fuses with distal tubular cells and will eventually give rise to the renal collecting ducts and to the ureter, which drains urine from the kidney. While the ureteric bud is connecting to distal tubules, newly differentiated cells (podocytes, endothelial cells, mesangial cells) participate in developing the glomerulus located adjacent to proximal tubule cells. Collectively, this developmental process leads to the development of the nephron, the functional unit of the kidney, and is termed nephrogenesis

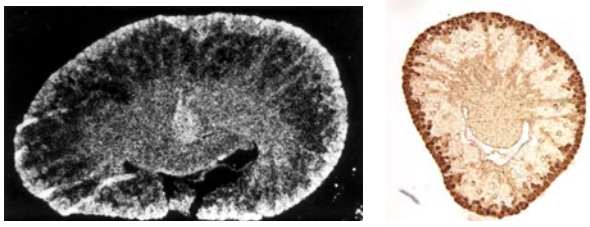


Figure 1-11. Cux-1 is highly expressed in the nephrogenic zone where cells are proliferating. (A) in situ hybridization of wild-type postnatal day 3 kidney shows that Cux-1 mRNA is highly expressed along the outer edges of the kidney in the nephrogenic zone. (B) Cells are undergoing cell proliferation in the outer edges of the kidney as indicated with PCNA labeling.

Murine nephrogenesis, the synthesis of new nephrons, begins at embryonic day 11.5 (E11.5) and continues until postnatal day 7 (P7). Cux-1 is highly expressed in proliferating cells of the nephrogenic zone (20) where the formation of new nephrons takes place. In our early studies, we demonstrated that maximum Cux-1 expression is detected early (E13.5) during nephrogenesis and then diminishes to basal levels towards the completion of nephrogenesis. Cux-1 is highly expressed in the nephrogenic zone, where cells are dividing and the synthesis of new nephrons is occurring (**Figure 1-11**). Since Cux-1 is expressed only in proliferating cells and not differentiated cells during nephrogenesis, we proposed that Cux-1 may act as a terminal repressor of differentiation. It is then plausible that abnormal expression and/or activation of *cux-1* during early renal development and/or in mature kidneys may produce aberrant cell proliferation. In the developing kidney, Cux-1 expression overlaps with that of the Notch pathway components in the condensing mesenchyme, in pretubular aggregates (comma and S-shaped bodies), and in the presumptive podocytes of capillary loop stage glomeruli (70). Our laboratory is currently investigating if Cux-1 expression is regulated by the Notch signaling pathway during early kidney development.

Overexpression of Cux-1 in CMV/Cux-1 transgenic mice resulted in kidneys that were enlarged 50% by 6 weeks of age, with the increased growth primarily restricted to the renal cortex (20). Proliferating cells were found in proximal and distal tubule epithelium throughout the cortex, and the squamous epithelium that normally lines Bowman's capsule was replaced with proximal tubule epithelium.

CMV/Cux-1 transgenic kidneys exhibited nephrons with lengthened tubules. However, the total number of nephrons was not increased. In the developing kidneys of transgenic mice, Cux-1 was ectopically expressed in more highly differentiated tubules and glomeruli, and this was associated with reduced expression of the cyclin kinase inhibitor, p27. Transient transfection experiments revealed that Cux-1 is an inhibitor of p27 promoter activity (20). These results suggest that Cux-1 regulates cell proliferation during early nephrogenesis by inhibiting expression of p27. Therefore, given the nature of Cux-1 expression during nephrogenesis and the expression pattern of p27, our lab has proposed a model for the function of Cux-1 during kidney development (**Figure 1-12**). Recently, we have demonstrated that Cux-1 binds to the p27 promoter at a region where sp1 is known to bind. Therefore, our model for Cux-1 function during nephrogenesis is that during early renal development when Cux-1 expression is elevated, Cux-1 in a concentration dependent manner can out compete sp1 transcription factor for promoter occupancy and repress p27 expression resulting in the increased cell proliferation occurring during early nephrogenesis. In contrast, during late nephrogenesis, when Cux-1 is expressed at relatively lower levels, sp1 can then outcompete Cux-1 for promoter occupancy resulting in the activation of p27 promoter and the inhibition of cell proliferation leading to the increase in differentiation that is observed at the conclusion of nephrogenesis. Evidence to date has demonstrated that Cux-1 is involved in regulating cell proliferation during kidney organogenesis (19, 20).

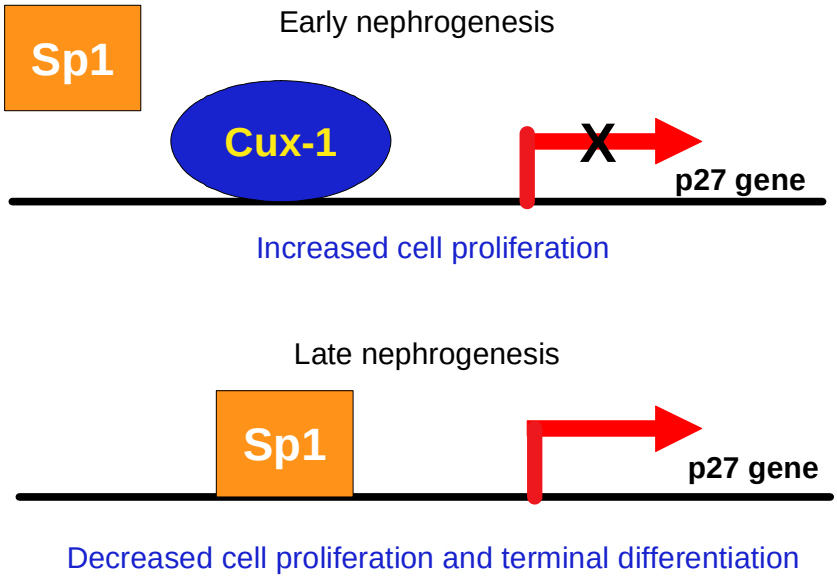


Figure 1-12. Model for Cux-1 function during nephrogenesis. During early stages of nephrogenesis when Cux-1 levels are elevated, Cux-1 may out compete Sp1 transcription factor for p27 promoter occupancy of a GC box resulting in repression of p27 and increased cell proliferation. In contrast, during the later stages of nephrogenesis when Cux-1 levels are attenuated, Sp1 may bind in a concentration dependent manner to p27 promoter leading to p27 expression and decreased progression through the cell cycle. Image taken from Alcalay et al. (241).

Polycystic Kidney Disease

Polycystic Kidney Disease (PKD) is one of the most common genetic, life threatening disease. PKD occurs in humans regardless of sex, age, race or ethnicity. PKD is characterized by enlarged bilateral cystic kidneys. The growth of many large epithelial-lined cysts from nephron collecting ducts results in polycystic kidneys. Human PKD can be inherited as an autosomal dominant (AD) or autosomal recessive (AR) trait. ADPKD and ARPKD result from mutations in a single gene. ADPKD occurs in 1:1000 individuals and occurs primarily as a result of mutations in

one of two genes, *PKD1* or *PKD2* (90). Mutations in *PKD1* located on human chromosome 16 are associated with 85% of all cases, whereas mutations in *PKD2* on chromosome 4 represent all other cases (91-98). ARPKD (1:20,000 live births) occurs primarily from mutations in a single gene, *PKHD1* (polycystic kidney and hepatic disease 1), which is located on chromosome 6 (96). *PKD1* encodes an 11 transmembrane G-coupled receptor termed Polycystin-1 (99) while *PKD2* encodes a Calcium channel named Polycystin-2 (100) (**Figure 1-13**).

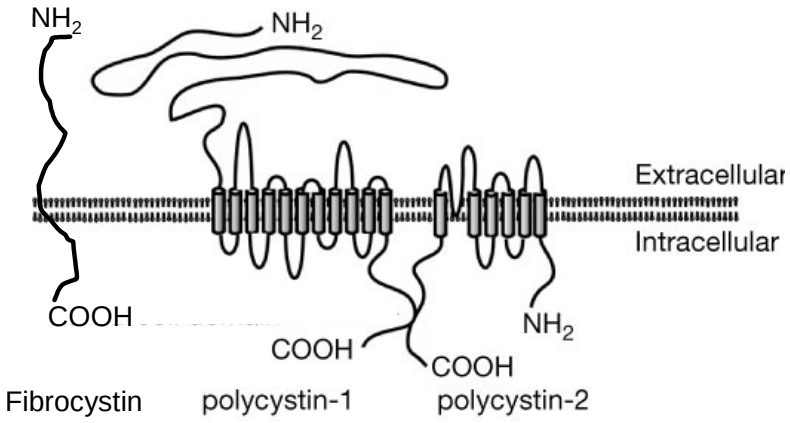


Figure 1-13 . Gene products of human polycystic kidney disease mutations. Polycystin-1 and -2 are associated with ADKPD, whereas Fibrocystin is the protein mutated in ARPKD.

Animal models have proven to be useful tools to examine human PKD. Murine PKD models have been demonstrated to have comparable cyst morphology, cyst localization, and disease progression (101). The congenital polycystic kidney (*cpk*) C57BL/6J (B6) mouse is the most extensively characterized PKD model. The *cpk* gene mutation is a spontaneous mutation within chromosome 12 on the B6 strain background (102). The cystic phenotype of *cpk* homozygotes appears to resemble

human ARPKD (103, 104). Mutation within *PKHD1* (Polycystic kidney hepatic disease-1) gene results in human ARPKD. *PKHD1* encodes a protein named Fibrocystin whose function is still not clear. However, Fibrocystin seems to function similar to the Notch and Polycystin-1 system in that the intracellular domain can localize to the nucleus (105, 106). Similar to the *PKHD1* mutation in humans, ARPKD progression in *cpk* kidneys is biphasic. Transient proximal tubule cystic dilations occur during early development followed by a transition to collecting duct cysts sometime during the first post-natal week of life as disease progresses (107). By 3 weeks of age, affected *cpk* mice have kidneys that are 10 times normal size and the mice die due to renal failure usually during the fourth week. The *cpk* gene is predicted to encode a novel 145 amino acid hydrophilic protein with no significant similarity to previously characterized proteins or protein domains (101). It has been suggested that *cpk* is an inactivating mutation that disrupts tubulo-epithelial differentiation in the kidney (108). However, the function of the *cpk* gene product (cystin) is unknown. Cystin is similar to gene products mutated in human PKD in that Polyductin, Polycystin-1 and Polycystin-2 localize to the primary apical cilia of tubular epithelial cells (**Figure 1-14**) (109,110). The similar localization of cystin, polyductin and the polycystins suggest convergence of similar pathways where mutation leads to cyst formation.

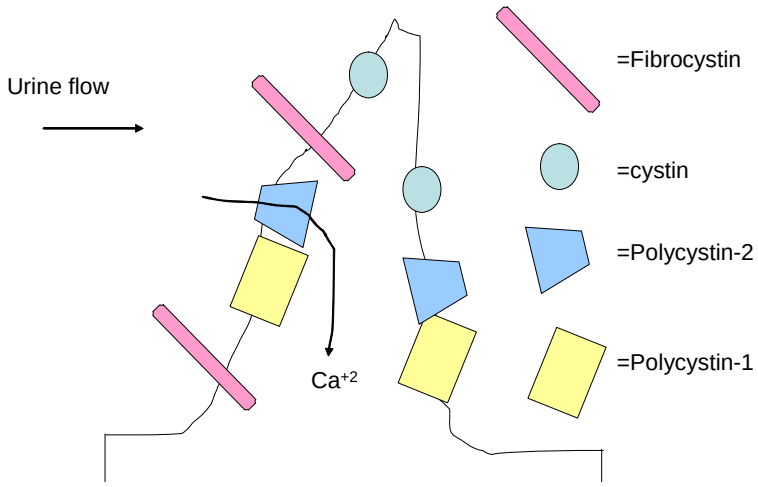


Figure 1-14. Gene products of PKD in humans and mice co-localize. Polycystins-1 and -2, Fibrocystin and cystin localize to primary apical cilia of nephron tubule epithelial cells

The mechanisms involving cyst development and progression within PKD are unclear. ADPKD patients carry one mutant allele of either the *PKD1* or *PKD2* gene. There has been evidence suggesting that a "second hit" may occur within discrete nephron tubular epithelium leading to dedifferentiation and aberrant cell proliferation (111). It is evident that cyst formation is a complex multi-factorial process involving disruption in planar cell polarity (112), increased cell proliferation (113) and increased fluid secretion (114, 115). ADPKD and ARPKD patients inheriting similar genetic mutations within the same family have highly variable phenotypes (116, 117). Moreover, murine models for PKD carrying identical genetic mutations have been found to posses different phenotypes depending upon the genetic strain background in which the PKD mutant gene is placed (101). Therefore, it has been suggested that the observed alterations in PKD phenotypes may be the result of the presence of modifying genes.

Modifier Genes

Modifying genes might cause more extreme (enhanced) phenotypes, less extreme (reduced) phenotypes, novel (synthetic phenotypes), or wild-type (normal) phenotypes (Reviewed in 117). Modification occurs when mutation of one gene alters the phenotype of another gene (118, 119).

There are two strategies for determining a candidate modifying gene; a "forward genetics" or "reverse genetics" approach (**Figure 1-16A**). Forward genetics is performed when a phenotype is identified and a genotype is unknown. Murine PKD models on inbred strain genetic backgrounds are very useful because they exhibit a specific phenotype that can be modified when crossed with another genetic background. For example, the *cpk* gene, when crossed onto different genetic backgrounds (CD1, Balb/c, DBA/2J, CAST/Ei) results in an altered phenotype (pancreatic and biliary ductal cysts) compared to the B6 background from which it originated (103,104, 108). The authors concluded that phenotypic differences between strains may be attributed to the presence of modifying genes.

Phenotype modification is identified when extreme phenotypes are observed (**Figure 1-15B**). In the case of PKD, modification is clear if the disease is ameliorated or exacerbated. For example, cystic kidney weight is used as a phenotypic marker to indicate PKD severity (103, 104, 120, 121). Populations of animals carrying the same extreme phenotype can be grouped together and analyzed for genetic similarity by positional cloning. Genetic regions observed in large numbers of animals with the identical extreme phenotype (negative or positive) are

termed quantitative trait loci (QTL). The hallmark of this forward genetics approach is that candidate modifying genes, located within QTL, can be identified when the locus is mapped.

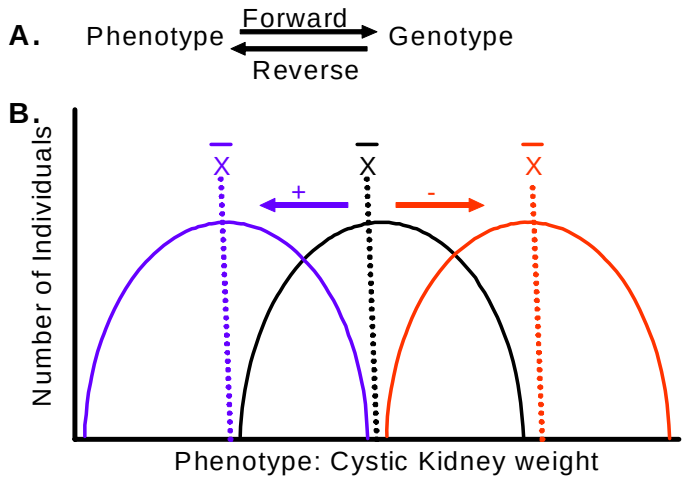


Figure 1-15. Identification of modifying genes. (A). A forward genetics approach is performed when the phenotype is known. Positional cloning is then utilized to identify candidate modifying genes located with a QTL. A reverse genetics approach is performed when a candidate modifier gene is identified. A candidate modifier gene is then confiemed as a modifier gene when it alters an established phenotype. (B) A phenotype can be quantified as the mean representation of a population. If the candidate modifying gene alters the mean phenotype negatively (red) or positively (blue), the candidate gene is then confirmed to be a modifying gene.

A disadvantage of the forward genetics approach is that genes located within the QTL need to be fully characterized. This amounts to an exhaustive procedure that first requires defining the function of each QTL gene. The generation of knockout and/or transgenic mice of each QTL gene may be required. Extensive functional analysis of a gene product will then determine if the gene is in the pathway of the PKD gene. Another disadvantage of forward genetics is that once a QTL gene function is determined to be involved within the pathway, the gene will

need to be mutated in animals to verify that the mutant gene itself does not produce an abnormal phenotype. An abnormal phenotype may contribute to the PKD phenotype and will qualify as an additive mutation instead of a modification. After this step has been accomplished, a reverse genetics approach is then required to demonstrate that a candidate gene is a modifying gene. Reverse genetics is performed when a genotype is known but the phenotype is unknown. Typically, to determine a modifying gene for PKD, a QTL candidate modifier gene is introduced with the PKD gene on the same strain genetic background (termed congenic). If the PKD phenotype is altered then the gene is confirmed as a modifying gene. So far many QTL have been identified in PKD. To date many QTLs have been identified (122-128), however no candidate modifying genes have been confirmed to modify PKD. This may be due to the complication in characterizing a QTL gene, generating a mutation within the gene that still carries a normal phenotype and then confirming modification by introducing the mutation onto mice with PKD.

Modifier genes in Polycystic Kidney Disease

Modifying genes are thought to contribute to the variable phenotypes observed in PKD patients (101, 129). Murine PKD models are convenient for analyzing mutations that modify cystic phenotype. Mice can be inbred containing identical genetic backgrounds. This permits a controlled environment whereby factors (i.e. food intake, genetic heterogeneity) that interfere with the determination of a modifying gene are reduced.

The murine *cpk* and oak-ridge polycystic kidney (*orpkd*) models for ARPKD have cystic kidneys where epidermal growth factor receptor (EGFR) tyrosine kinase activity is elevated (130). Similar to the *cpk* gene product cystin, the *orpkd* gene product polaris is located in primary apical cilia (131). In both models and in human ARPKD, EGFR was found to have increased tyrosine kinase activity (130). EGFR is normally localized specifically to the basolateral surface of renal epithelial cells lining collecting tubules (132). However, in humans with ARPKD and in animal models for ARPKD (*cpk and orpkd*) a phenomenon occurs whereby cystic cells are thought to have altered cellular polarity (131, 132). This is thought to contribute to the mislocalization of EGFR to the apical membrane.

The *wa-2* point mutation (valine→glycine at position 743) in EGFR decreases tyrosine kinase activity (133). Mislocalization of EGFR to the apical membrane does not affect the *wa-2* mutation in reducing EGFR activity (132, 131). Richards et al. (131) demonstrated that *wa-2* improves kidney function associated with decreased cyst formation in *orpkd.* The authors found the *wa-2* mutation is most potent in reducing cysts in collecting ducts where EGFR is mislocalized to the apical surface. In contrast, cysts remained present in *wa-2/orpkd* proximal tubules where EGFR is not mislocalized to the apical surface. These results suggest modification of cyst formation requires both EGFR mislocalization and *wa-2* mutation. Therefore, *wa-2* is not considered a modifier gene of *orpkd* because modification does not exclusively require the *wa-2* gene.

In general, a gene is defined as a modifier gene if its mutation alters the phenotype of another gene present in a pathway (134). The altered cellular polarity and mislocalization of EGFR (131, 132) is likely to be derived from a separate pathway caused by *orpkd* that does not involve the EGFR pathway. The *wa-2* mutation resulting in reduction of EGFR activity and decreased cyst formation requires both EGFR mislocalization and *wa-2*. Therefore, *wa-2* is not a modifier gene but instead is considered an additive mutation. An example of an additive mutation would be any mutation contributing to the PKD phenotype that is involved in a pathway separate from the PKD pathway. As a result of the development of mouse genetics involving knockout and transgenic technologies, additive mutations are relatively easier to determine than modifying genes.

Role of Cux-1 in Polycystic Kidney Disease

Cell proliferation is important in polycystic kidney disease. Cyst-lining epithelial cells abnormally proliferate in rodents and humans. Rodents have proven to be very useful tools to analyze human disease. Murine PKD models have been described in which the mutant phenotype closely resembles human PKD with respect to cyst morphology, cyst localization, and disease progression (reviewed in 118, 119). Since the most extensively characterized PKD model is the congenital polycystic kidney (*cpk*) C57BL/6J (B6) mouse, Cux-1 expression levels within *cpk* kidneys were evaluated. Cux-1 mRNA and protein were ectopically expressed during the later stages of cystic disease (19, 135). Ectopic expression of Cux-1 in

cyst lining epithelial cells suggested that Cux-1 may play a role in polycystic kidney disease. In contrast, Cux-1 expression was restricted to interstitial cells during early stages in cpk cystic kidneys (**Figure 1-16**) (135).

Cux-1 expression in cyst-lining epithelial cells during late stages of PKD, co-localized with PCNA while also staining positive for TUNEL (135). Since Cux-1 normally co-localizes with PCNA positive cells that are proliferating during kidney development and cystic epithelial cells are dedifferentiated and rapidly divide, the

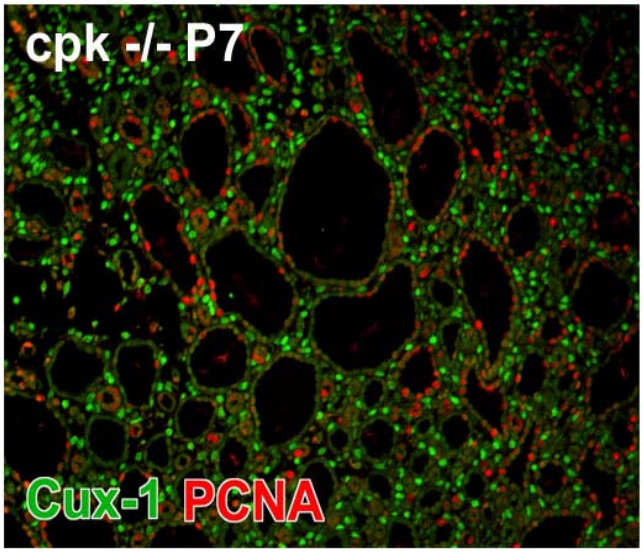


Figure 1-16. Differential expression of Cux-1 and PCNA in the cysts of 7 day old *cpk* kidneys. 7 day old *cpk* kidneys were labeled for Cux-1 (green) and PCNA (red). Cux-1 and PCNA co-calize in the nephrogenic zone, but not in the medulla where cysts are forming. Cells lining the cysts are PCNA positive while Cux-1 expression is restricted to interstitial cells. Image taken from Sharma et al. (135).

coexpression of Cux-1 in cystic epithelial cells was not surprising. Cystic epithelial cells undergo apoptosis during the later stages of *cpk* cystic disease (135, 136). Apoptosis occurring within *cpk* cyst-lining epithelial cells may occur as a result of cyst expansion leading to the destruction of nephrons. Moreover, apoptosis within cystic epithelial cells may serve as a compensatory mechanism to eliminate cells engaged in aberrant cell proliferation pathways. Ostrom et al. (137) demonstrated that reduced *Pax2* gene dosage increases apoptosis and slows the progression of renal cystic disease. The involvement of Cux-1 in apoptotic pathways within cyst-lining cells and polycystic kidneys is currently unknown.

Pkd1 null mice develop polycystic kidneys during embryogenesis (138). We found that Cux-1 is ectopically expressed in cyst-lining epithelial cells within Pkd1 null kidneys (135). In both early and late embryonic kidneys from *Pkd1* null mice, Cux-1 was highly and ectopically expressed in normal-appearing tubule epithelium, interstitial cells, and in the epithelial cells lining the cysts, where it co-localized with proliferating cell nuclear antigen (PCNA). Increased Cux-1 expression in *Pkd1* null kidneys was also associated with a decrease in p27 expression. In contrast, in *cpk* kidneys p27 levels were found to be highly expressed (**Figure 1-17**) (135). Upregulation of p27 levels may be a consequence of high TGFβ levels which progressively increase with age in *cpk* kidneys and may induce cyclin kinase

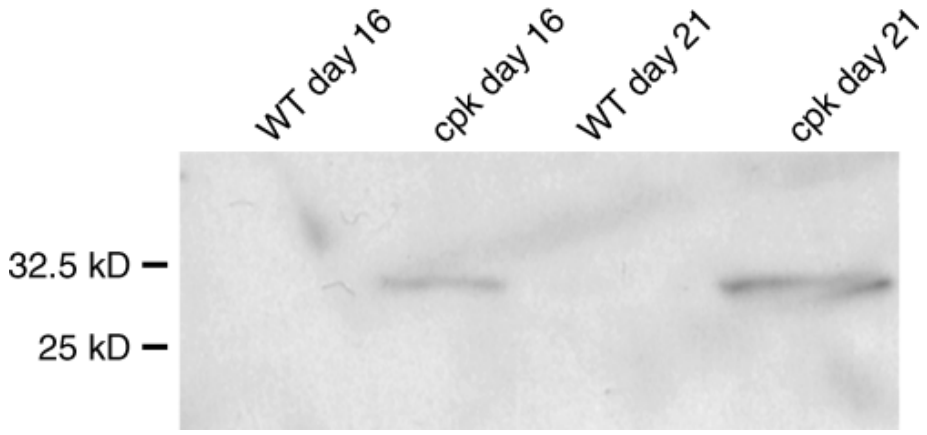


Figure 1-17. p21 and p27 protein expression in *cpk* kidneys. The cyclin kinase inhibitor p27 is highly expressed during the late stages of *cpk* cystogenesis. Image taken from Sharma et al. (135).

inhibitor upregulation (139). During the later stages of cystogenesis, Cux-1 and p21 were co-localized in cyst lining cells (135). These results suggest that cyst development in *Pkd1* null mice and *cpk* mice proceeds through different mechanisms. In *Pkd1* null mice, ectopic expression of Cux-1 is associated with increased cell proliferation (135). In contrast, in *cpk* mice, ectopic expression of Cux-1 is associated with apoptosis (135). A correlation between increased apoptosis and *cpk* disease progression has been demonstrated (135, 136, 140). Since Cux-1 becomes ectopically expressed only during later stages in *cpk* mice and is present within cells undergoing apoptosis (135, 136), it is possible that Cux-1 becomes upregulated when cells undergo apoptosis. Overexpression of Cux-1 alone does not lead to polycystic kidneys. Transgenic mice that overexpress Cux-1 driven by the CMV promoter have organs that are hyperplastic (20). The ectopic expression of

Cux-1 within *cpk* cyst lining cells may be a consequence of those cells becoming dedifferentiated. Cux-1 may be induced by altered signaling pathways within dedifferentiated cyst lining epithelial cells as these cells become programmed to enter the cell cycle and divide. It is also possible that Cux-1 participates in pathways that indirectly contribute to the overall cystic phenotype. The role of Cux-1 in the *cpk* mouse model for ARPKD is not clear.

Cux-1 participation in pathways involved in cyst formation and progression occurring with PKD are not known. The epidermal growth factor pathway has been suggested as a pathway that participates in the severity of *cpk* polycystic kidney disease (130-132, 141) Point mutation within the epidermal growth factor receptor (EGFR) results in mice with wavy hair, curly whiskers and a lactation defect (133) which is the exact same phenotype observed in mice carrying a 246 amino acid deletion of Cux-1 (termed *cux-1ΔCR1*) (37). This suggests that Cux-1 might be involved in the EGFR pathway.

The role of cyclin kinase inhibitors (CKI) and aberrant cell-cycle regulation occurring within Polycystic Kidney diseases has become well established with CKI being targeted as a potential treatment (142-147). Cux-1 is highly expressed during G1-S phase transition and regulates the cell cycle by repressing the cyclin kinase inhibitors p21 and p27. Bhunia et al. (148), showed that expression of polycystin-1 activates the JAK-STAT pathway, thereby upregulating p21 (waf1) and inducing cell cycle arrest in G0/G1. This process requires Polycystin-2, a channel protein, as an essential cofactor. Mutations that disrupt polycystin-1/2 binding prevent activation

of the pathway (98, 102, 149). Mouse embryos lacking Pkd1 have defective STAT1 phosphorylation and p21(waf1) induction (148). These results suggest that one function of the polycystin-1/2 complex is to regulate the JAK/STAT pathway which may help to explain how mutation in either gene can result in deregulated growth.

The observation that abnormal cell-cycle regulation is occurring in PKD (142-147) is further supported by Li et.(142) who showed that the ADPKD protein polycystin-2 (PC2) regulates the cell cycle through direct interaction with Id2, a member of the helix-loop-helix (HLH) protein family that is known to regulate cell proliferation and differentiation. Since Cux-1 represses the cyclin kinase inhibitor p21 (55) and PKD1 is involved in the JAK STAT pathway where p21 is upregulated (148), our laboratory has proposed a model (**Figure 1-18**) for Cux-1 and p21 expression.

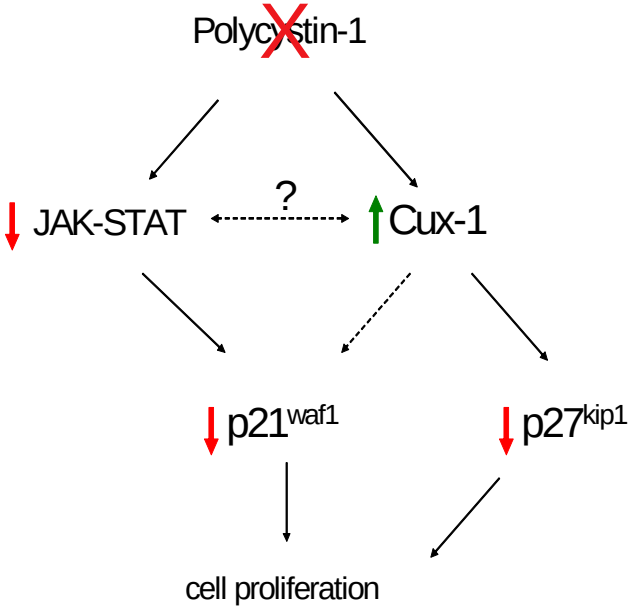


Figure 1-18. Model for Cux-1 function in ADPKD. Mutation in Polycystin-1 results in decreased JAK-STAT signaling and reduced p21 expression which favors rapid progression through the cell cycle leading towards aberrant cell proliferation. Mutation in Polycystin-1 results in the ectopic expression of Cux-1 directly or indirectly related to the JAK-STAT pathway. Cux-1 is known to repress the cyclin kinase inhibitors p21 and p27 which would favor aberrant cell proliferation occurring in ADPKD.

Cux-1 function is regulated by phosphorylation and dephosphorylation. Phosphorylation of Cux-1 by Casein Kinase II (59) and PKC (60) restricts Cux-1 DNA binding ability whereas dephosphorylation by cdc25A phosphatase permits Cux-1 DNA binding ability (55). Casein Kinase II and PKC seem to participate in the Polycystin1/2 complex pathway. Casein Kinase II is involved in the phosphorylation of Polycystin-2 and modulates Polycystin-2 calcium channel activity (150, 151). In sensory cilia of Caenorhabditis elegans male-specific neurons, the *LOV-1* (*PKD-1*) and *PKD-2* genes are required for mating behavior(152, 153). Hu et al. (151) identified the regulatory subunit of the serine-theonine casein kinase II (CK2) as a binding partner of LOV-1 and human PC-1. They discovered that Casein Kinase II and the calcineurin phosphatase TAX-6 modulate male mating behavior and PKD-2 ciliary localization. The role of Casein Kinase II in PKD is still not clear.

PKC appears to play an important role in PKD pathways (154, 155). Polycystin 1 mediates PKC alpha-dependent and c-Jun N-terminal kinase-dependent activation of the transcription factor AP-1 (154). Arnould et al. (154, 155), found that PKD2-mediated AP-1 activity was dependent upon activation of the mitogen-activated protein kinases p38 and JNK1 and protein kinase C (PKC) epsilon, a calcium-independent PKC isozyme. Since Casein Kinase II (59) and PKC (60) have been demonstrated to phosphorylate Cux-1, it is possible that PKC and/or Casein Kinase II phosphorylation in PKD pathways might contribute to inhibition of Cux-1

function both directly or indirectly. The regulation of Cux-1 within cyst-lining epithelial cells may involve PKC and Casein Kinase II activity within those cells.

Several studies demonstrated that polycystin-1 is involved in heterotrimeric G protein signaling (156, 157). It has been shown that the Polycystin-1 C-terminus binds and activates heterotrimeric G proteins in vitro (156) and that the activation of JNK and AP-1 by polycystin-1 is mediated via Gα and Gβγ subunits (157). Stimulation of cAMP and MAP kinases results in pathways leading to cell proliferation. cAMP may have a central role in cyst growth by stimulating both fluid secretion and cell proliferation (158-161). Polycystin-1 C-tail has been implicated in regulating ERK activity (162). Moreover, Calcium restriction of human ADPKD cells results in cAMP activation of the b-RAF/ERK pathway switching cells that are not in a proliferative state to a cAMP dependent growth-stimulated phenotype (163). According to these studies, cultured cyst epithelial cells from ADPKD kidneys show increased rates of cell proliferation in response to cAMP, which activates B-Raf instead of inhibiting Raf-1. B-Raf then activates the MEK/ERK pathway and cell proliferation. This "PKD phenotype" is replicated in mouse M-1 cortical collecting duct cells stably transfected to overexpress of a short, polycystin-1 cytosolic C-terminal tail construct (164), suggesting that this cAMP-responsive PKD phenotype involves disruption of polycystin function in these cells. It is possible that Cux-1 may be an endpoint in cAMP and MAP kinase pathways given the expression of Cux-1 in proliferating cells. cAMP can activate the protein kinase A(PKA) pathway. Cux-1 is phosphorylated by cAMP stimulated protein kinase A (PKA)

activity which is dependent for cell proliferation and motility (61). Furthermore, upregulation of transforming growth factor beta (TGF-β) mRNA is found in PKD rodent models (139, 165). Since Cux-1 is a known target of TGF-β activity (166, 167) the TGF-β pathway is another pathway in PKD that might lead to Cux-1 activity. Given that aberrant cell proliferation is a hallmark of PKD and Polycystin-1 regulates p21 (148), a cyclin kinase inhibitor that is similarly targeted by Cux-1(55), there is a high possibility that Cux-1 may be involved in PKD. Therefore, the evidence to date suggests that it is very likely that Cux-1 could be involved in multifactorial signaling pathways in polycystic kidney diseases.

Goal of these studies

Cux-1 is involved in kidney development within drosophila and mice. The signaling pathways that may exist upstream and downstream of Cux-1 during kidney development have yet to be fully evaluated. Cux-1 is abnormally expressed in human and mouse models for polycystic kidney disease. However, it has never been demonstrated that Cux-1 overexpression or gene deletion alters the PKD phenotype. Therefore, the mechanisms by which Cux-1 may function in kidney development and polycystic kidney disease remain nebulous. The collective goal of this body of work was to **1) Determine if post-translational modification of Cux-1 affects kidney development, 2) Determine if Cux-1 can alter the PKD phenotype, and 3) Evaluate how Cux-1 may function within PKD.** Collectively, these studies

have revealed the importance of post-translational modification of Cux-1 during kidney development and polycystic kidney disease.

CHAPTER TWO

Effect of Cux-1 phosphorylation on Kidney Development

Abstract

Cux-1 is a murine homeobox gene structurally related to Drosophila cut. Cux-1 is highly expressed in the nephrogenic zone of the developing kidney, where its expression coincides with cell proliferation. Cux-1 functions as a transcriptional repressor of the cyclin kinase inhibitors (CKI) p21 and p27. Cux-1 DNA binding activity is negatively regulated by phosphorylation, and dephosphorylation of Cux-1 results in increased DNA binding. Transgenic mice ectopically expressing Cux-1 develop renal hyperplasia associated with the down-regulation of the CKI p27. Calcineurin A (CnA) α(-/-) mice display renal hypoplasia associated with the ectopic expression of p27. CnA is a serine/threonine phosphatase activated by intracellular calcium. Inhibiting CnA with cyclosporin A (CsA) leads to nephron deficit in rat metanephric organ cultures and apoptosis in various renal cell lines. To determine whether the ectopic expression of p27 in CnA-α -/- kidneys results from the down-regulation of Cux-1, metanephroi from embryonic Cux-1 transgenic and wild-type mice were harvested and cultured with CsA for 5 days. CsA treatment significantly inhibited growth of wild-type metanephroi. In contrast, CsA-treated Cux-1 transgenic kidney cultures were not growth inhibited, but showed high levels of cell proliferation in the nephrogenic zone. Moreover, in CsA-treated Cux-1 transgenic kidney cultures, p27 was not expressed in the nephrogenic zone, but only up-regulated in maturing glomeruli and tubules. Taken together, our results demonstrate

that ectopic expression of Cux-1 can rescue the effects of CsA inhibition of CnA and suggest that Cux-1 may be regulated by calcineurin A.

Introduction

Cux-1 is a murine homeobox gene that is structurally related to Drosophila cut. Mammalian homologues of cut function primarily as transcriptional repressors of many different genes, including genes encoding the cyclin kinase inhibitors (CKI) p21 and p27 (20, 55, 46, 168, 169). The binding of Cux-1 to the promoters of these genes appears to be limited to tissues or stages of development where the target genes are not expressed (13). At later stages of development, Cux-1 proteins are down-regulated or lose their ability to bind DNA, and transcription of the target genes is permitted. Cut proteins repress transcription by two different mechanisms: (1) passive repression, whereby cut proteins compete with transcriptional activators for the same binding site (known targets of passive repression by Cux-1 are Sp1 (GC box) and NF-Y (CCAAT box), or (2) active repression, by means of a carboxy-terminal domain after binding to DNA at a distance from the transcription start site (48).

During kidney development, Cux-1 is highly expressed in the nephrogenic zone, in uninduced and condensing mesenchyme, comma- and S-shaped bodies, and branching ureteric buds. At later stages of development, Cux-1 is down-regulated such that expression is minimal in maturing glomeruli and tubules (19). This finding is associated with the up-regulation of the cyclin kinase inhibitor p27 in maturing

glomeruli and tubules, which induces cell cycle arrest and terminal differentiation. Previously, we generated transgenic mice ectopically expressing *cux-1* under the direction of the CMV immediate early gene promoter. These mice express Cux-1 in maturing glomeruli and tubules in developing kidneys, resulting in renal hyperplasia, associated with the down-regulation of p27 (20).

Within the cut repeats, there are evolutionarily conserved consensus phosphorylation sites for protein kinase C (PKC) and casein kinase II (59, 60). Moreover, PKC and casein kinase II were shown to phosphorylate Cux-1 on specific serine and threonine residues in the cut repeats. Phosphorylation of Cux-1 resulted in an inhibition of DNA binding activity, and a concomitant inhibition of repression, while treatment with alkaline phosphatase restored DNA binding. Analysis of Cux-1 binding activity in vivo demonstrated that Cux-1 bound DNA when in a dephosphorylated state, but did not bind DNA when phosphorylated. These results demonstrated that Cux-1 activity is regulated by phosphorylation and suggests that specific phosphatases may play a role in Cux-1 activity.

Calcineurin is a SER/THR phosphatase that exists as a heterodimer of a catalytic subunit, called calcineurin A (CnA), and a regulatory subunit, called calcineurin B (CnB) for review see 170). Calcineurin phosphatase activity is initiated only when CnB is bound to both calmodulin and calcium. There are three isoforms of CnA, α, β, and γ, that display some tissue specificity. CnA-α and CnA-β are widely expressed, while the γisoform appears to be restricted to testis and brain (171). Mice carrying a targeted deletion of CnA-α exhibit severe defects in postnatal

kidney development, including reduced cell proliferation and increased apoptosis in the nephrogenic zone, contributing to an overall reduction of the nephrogenic zone (172). Additional changes include an absence of mesangial cells in the glomeruli that develop during postnatal kidney development. The cell cycle defect is thought to result from the ectopic expression of the cyclin kinase inhibitor p27 (172). In contrast, mice carrying a targeted deletion of CnA-β have normal kidneys (172). To determine whether the ectopic expression of p27 in the nephrogenic zone of CnA α(-/-) kidneys might result from the down-regulation of Cux-1, we examined Cux-1 expression in kidneys isolated from CnA-α null mice. To determine whether increased Cux-1 expression would rescue the cell proliferation defect observed in the kidneys of CnA-α null mice, we evaluated cell proliferation in kidney organ cultures from Cux-1 transgenic mice treated with cyclosporin A, a specific inhibitor of calcineurin.

Materials and Methods

Antibodies

Commercial reagents used were rabbit anti-CDP (Cux-1; Santa Cruz # sc-13024), rabbit anti-p27 (Santa Cruz #sc-528), mouse anti-PCNA (SIGMA # P-8825), and goat anti-Pax-2 (Santa Cruz #sc-7747).

Organotypic Kidney Cultures

Metanephric organ cultures were established from mouse embryos as described (173). Embryos were dissected from timed pregnant mice at 12.5 or 14.5 days post coitum (dpc). Embryonic age was verified according to Theiler (174). Fifteen paired metanephric kidneys and associated ureteric buds were microdissected from wild-type or Cux-1 transgenic mice and placed in a 24-well tissue culture plate containing medium (50/50 DMEM/F12, 2 mM L-glutamine, 10 mM Hepes, 5 g/ml insulin, 5 μg/ml transferrin, 2.8 nM selenium, 25 ng/ml prostaglandin E, 32 pg/ml T3, and 250 μg/ml). Kidneys were placed on 0.4 μM PET track-etched membranes (Beckton Dickinson) in medium for 24 hr and then replaced with medium containing either 500 ng/ml CsA or vehicle (100% ethanol). Organ cultures were replenished with fresh treatment medium every 24 hr and photographed using a Leica M240 microscope and captured with an Optronics Magnafire digital camera.

Preparation of Phosphoproteins and Western Blot Analysis

Total protein lysates from 15 paired wild-type metanephric kidney cultures grown in media containing either 500 ng/ml cyclosporin A or vehicle (100% ethanol) were applied to PhosphoProtein purification columns using the QIAGEN PhosphoProtein Purification Kit, according to the manufacturer's directions. In some cases, lysates were first incubated with calf intestinal phosphatase (5 μg protein/2 units). Total protein lysates or purified phosphorylated proteins (42 μg) were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample

buffer and electrophoresed on 4-15% gradient polyacrylamide gels. Phosphoproteins were transferred to a polyvinylidene difluoride membrane as described previously (20). The immunoblot was blocked in 5% bovine serum albumin in TBST buffer (10 mM Tris-Cl; 150 mM NaCl, pH 7.5; 0.1% Tween 20). Primary antibodies were added at a dilution of 1:100 for Cux-1, PhosphoSerine, and PhosphoThreonine in blocking solution. After overnight incubation at 4°C, filters were washed twice at room temperature with TBS-Tween/Triton buffer (20 mM Tris-Cl; 500 mM NaCl; 0.05% Tween 20; 0.2% Triton X-100, pH 7.5), washed twice at room temperature in TBS buffer, and incubated with secondary antibodies (1:10,000 dilution in TBST) for 1 hr at room temperature. After four additional washes in TBS-Tween/Triton buffer, bound antibody was detected by Super Signal West Pico chemiluminescent Substrate (Pierce) according to manufacturer's directions, followed by exposure to autoradiography film. Total Cux-1 and phospho-Cux-1 protein levels were scanned and quantitated using Gelpro 4.5 software (Media Cybernetics).

Morphologic Studies

For quantitation of metanephric size, kidney organ cultures from wild-type or Cux-1 transgenic mice, treated with cyclosporin A or vehicle, were photographed under light microscopy at identical magnification. Organ culture pictures were quantified using the NIH image J program. Metanephroi were outlined manually, and the surface areas were calculated by pixel counting. Net growth was determined as the mean percentage of net surface area before treatment (baseline) subtracted from

net surface area after treatment. Collective repeated measures analysis of variance (ANOVA) was performed during treatment with one-way ANOVA performed on each treatment day to determine a difference between treatment groups. $P \leq 0.05$ was considered as a difference in treatment and was followed with post hoc analysis by the Least Significant Difference test, where $P \leq 0.05$ again was considered statistically significant.

Immunofluorescence

Metanephroi were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer-thick tissue sections were deparaffinized with xylene and rehydrated with graded ethanols. To obtain adequate signal, the slides were treated with antigen unmasking solution (Vector) according to the manufacturer's protocol. To block endogenous autofluorescence, sections were incubated with 1 M NH_4Cl for 30 min. Sections were washed in phosphate buffered saline (PBS) and blocked with 10% normal serum (in PBS from the species the secondary antibody was raised in) for 1 hr. After washing in PBS, the slides were incubated for 1 hr with primary antibodies in a humidified chamber. Antibody dilutions were 1:50 for CDP Ab, 1:3,000 for PCNA Ab, and 1:100 for p27 Ab, in 2% blocking serum in PBS. Slides were incubated at room temperature with 100 µl of primary antibody in a humid chamber and then washed 4 times in PBST. For Cux-1 and PCNA double-labeling experiments, sections were washed with PBST and incubated simultaneously with biotin-conjugated horse anti-rabbit and Texas-Red conjugated horse anti-mouse

secondary antibodies (Vector). Sections were then washed with PBST and incubated with fluorescein isothiocyanate-conjugated avidin (Vector). Sections were then washed, mounted with Vectashield (Vector), and viewed with a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera.

Results

To determine whether the reduced nephrogenic zone and increased expression of p27 in CnA α(-/-) mice resulted from alterations in Cux-1 expression or activity, we examined Cux-1 expression in kidneys isolated from CnA α(-/-) mice. In kidneys isolated from 4-day-old wild-type mice, Cux-1 was highly expressed in the nephrogenic zone where it colocalized with proliferating cell nuclear antigen (PCNA; **Figure 2-1B-D**). In kidneys isolated from CnA α(-/-) mice, Cux-1 continued to be expressed in the nephrogenic zone (**Figure 2-1F**). However, in contrast to wild-type, PCNA was expressed in far fewer cells in CnA α(-/-) kidneys and did not colocalize with Cux-1 (**Figure 2-1G,H**). Previous studies have shown that the DNA binding activity of Cux-1 is regulated by PKC and casein kinase II (59, 60). DNA binding activity was reduced in the presence of PMA, an activator of PKC, but mutation of the PKC sites resulted in DNA binding that was not reduced by PMA treatment. One possibility is that the absence of CnA αresults in Cux-1 being maintained in the phosphorylated inactive form and suggests that CnA αmay positively regulate Cux-1 activity.

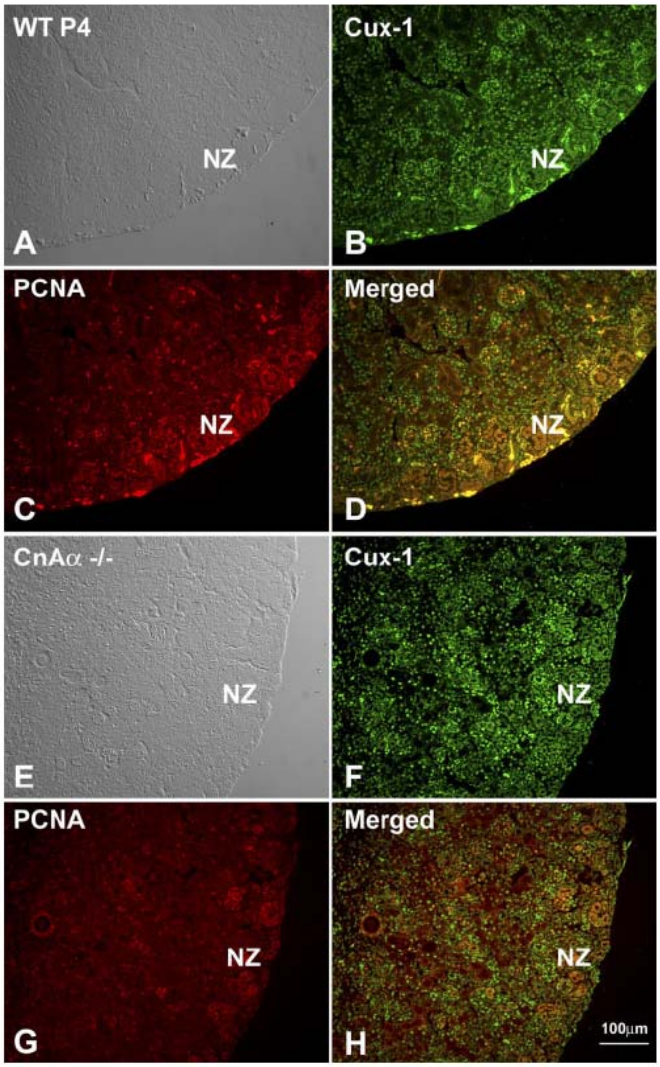


Figure 2-1. Expression of Cux-1 in kidneys from CnA-α -/- mice. A-H: Four-day-old kidneys from wild-type (A-D) and Calcineurin A (CnA) -α -/- (E-H) mice were labeled with antibodies against Cux-1 (B,F) and proliferating cell nuclear antigen (PCNA, C,G). D: The merged image shows that Cux-1- and PCNA-expressing cells were colocalized in the nephrogenic zone of the wild-type kidneys. H: In contrast, there were fewer PCNA-positive cells in the CnA-α -/- kidneys, and Cux-1-expressing cells did not colocalize with PCNA. NZ, nephrogenic zone. Scale bar = 100 μm. Image taken from Alcalay et al. (242).

The antibiotic cyclosporin A (CsA) competitively binds calcineurin to inactivate its phosphatase activity (175). Previous studies have shown that inhibiting CnA with CsA leads to nephron deficit in rat metanephric organ cultures (176). **Figure 2-2** shows that metanephroi grown in the presence of CsA continued to express Cux-1, but the expression of PCNA was markedly reduced. We next examined the expression of Pax-2, a marker for condensing mesenchyme and early nephric structures in the nephrogenic zone, and p27, a marker for maturing glomeruli and tubules, in the vehicle (control) and CsA-treated kidney organ cultures. Figure 3 shows that Pax-2 was expressed in the nephrogenic zone of vehicle-treated metanephroi, while p27 was expressed in maturing glomeruli and tubules in a region characterized by the absence of Pax-2. In contrast to the control kidney, the region of Pax-2 expression was significantly smaller in the CsA-treated metanephroi (Fig. 3F). Moreover, p27-positive cells were found at the periphery of the developing kidney culture and within the Pax-2-positive cells (**Figure 2-3G,H**). These results indicate that CsA treatment results in a reduction in the nephrogenic zone and in ectopic expression of p27 and demonstrate that CsA treatment of metanephroi in organ culture phenocopied the CnA α(-/-) phenotype. Thus to begin to determine whether the ectopic expression of p27 in the nephrogenic zone of CnA α(-/-) kidneys results from the down-regulation of Cux-1 activity, metanephric kidneys isolated from transgenic and wild-type mice were treated with cyclosporin A. CsA- or vehicle-treated metanephroi were examined daily by light microscopy. Initially, all wild-type metanephroi were matched for gestational age and had a similar

appearance (**Figure 2-4A,G**). By 24 hr, the CsA-treated metanephroi were visibly smaller than the vehicle-treated metanephroi (**Figure 2-4B,H**). While the vehicle-treated metanephroi continued to increase in surface area, the CsA-treated metanephroi were growth inhibited (**Figure 2-4A-L**). Similar to wild-type kidneys, all Cux-1 transgenic metanephroi were matched for gestational age and had a similar appearance (**Figure 2-4 M,S**). However, in contrast to wild-type kidneys, metanephroi isolated from Cux-1 transgenic mice continued to grow when treated with CsA (**Figure 2-4M-X**).

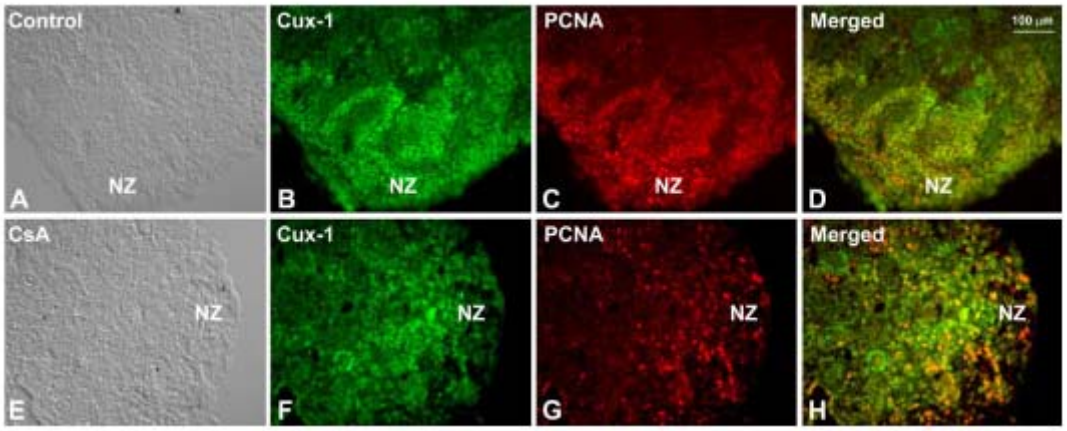


Figure 2-2. Decreased cell proliferation in cyclosporin-treated kidney organ cultures from wild-type mice. A-H: Sections of kidney organ cultures treated with vehicle (control, A-D) or cyclosporin A (CsA, E-H) for 5 days were labeled with antibodies against Cux-1 (B,F) and proliferating cell nuclear antigen (PCNA, C,G). D: The merged image shows that Cux-1 and PCNA were coexpressed in the vehicle-treated cultures, in regions corresponding to the nephrogenic zone. H: Similar to kidneys from CnA-α -/- mice, there were fewer PCNA-positive cells in the CsA-treated kidney cultures. NZ, nephrogenic zone. Scale bar = 100 μm. Image taken from Alcalay et al. (242).

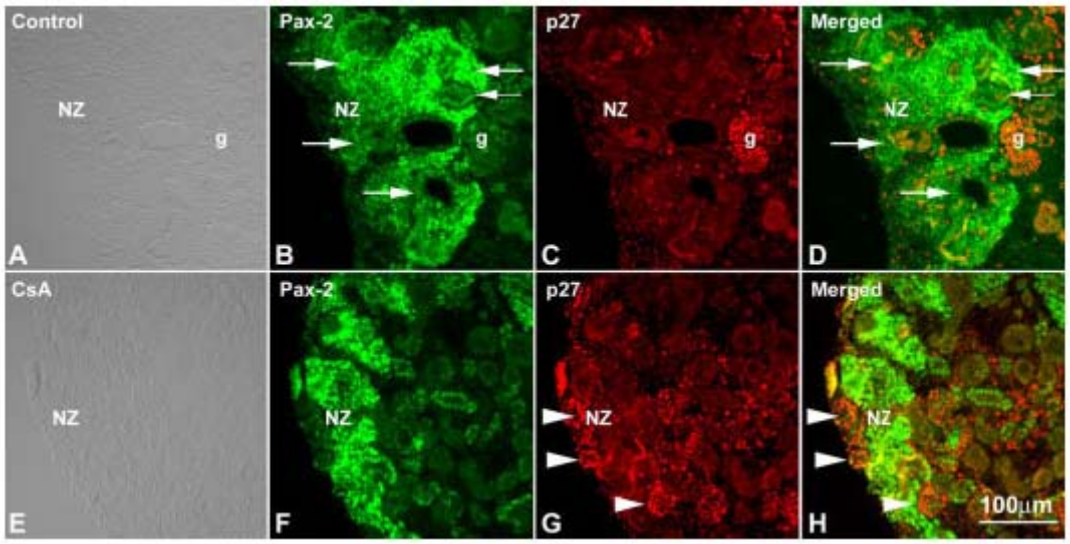


Figure 2-3. Ectopic expression of p27 in cyclosporin-treated kidney organ cultures from wild-type mice. A-H: Sections of kidney organ cultures treated with vehicle (control, A-D) or cyclosporin A (CsA, E-H) for 5 days were labeled with antibodies against Pax-2 (B,F) and p27 (C,G). B-D: Pax-2 is expressed in condensed mesenchyme and early nephric figures in the nephrogenic zone of control kidney cultures (arrows, B,D). C,D: In contrast, p27 is expressed in maturing glomeruli (g) and tubules and is excluded from the nephrogenic zone in vehicle-treated cultures. F-H: In the CsA-treated kidney cultures, the region of Pax-2 expression was reduced, corresponding to a decrease in the nephrogenic zone (F,H). G,H: In addition, p27 was ectopically expressed at the periphery of the kidney culture in the nephrogenic zone (arrowheads). This finding is similar to the previously described ectopic expression of p27 in the CnA-α -/- mice (172). NZ, nephrogenic zone; g,h glomerulus. Scale bar = 100 μm. Image taken from Alcalay et al. (242).

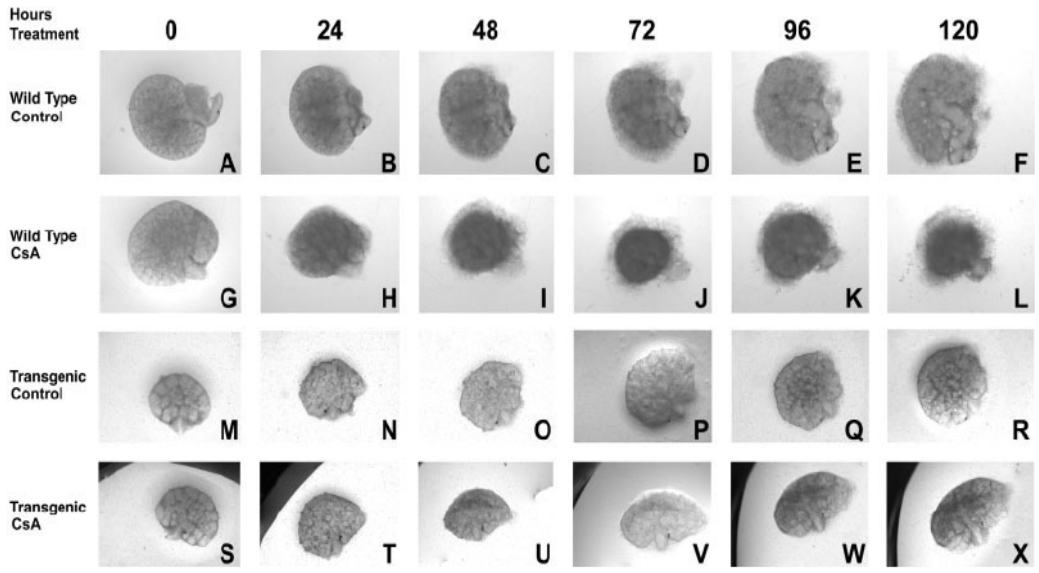


Figure 2-4. Morphology of metanephric kidneys cultured in the presence of cyclosporin A. A-X: Metanephroi were harvested from embryonic wild-type (A-L) or Cux-1 transgenic (M-X) mice and cultured in the presence of cyclosporin (G-L,S-X) or vehicle (A-F,M-R). Serial light micrographs of paired metanephroi are shown. B-F: Metanephroi from wild-type mice treated with vehicle (control) showed significant growth and differentiation after growth for 24 (B), 48 (C), 72 (D), 96 (E), and 120 hr (F). H-L: Metanephroi from wild-type mice treated with 500 ng/ml cyclosporin A showed some growth inhibition after 24 hr (H) and were severely growth inhibited after 48 (I), 72 (J), 96 (K), and 120 hr (L) of treatment. M-X: In contrast, both vehicle (M-R) and cyclosporin A (S-X) Cux-1 transgenic kidney cultures showed extensive growth and differentiation after 24 (N,T), 48 (O,U), 72 (P,V), 96 (Q,W), and 120 hr (R,X) treatment with 500 ng/ml cyclosporin A. Image taken from Alcalay et al. (242).

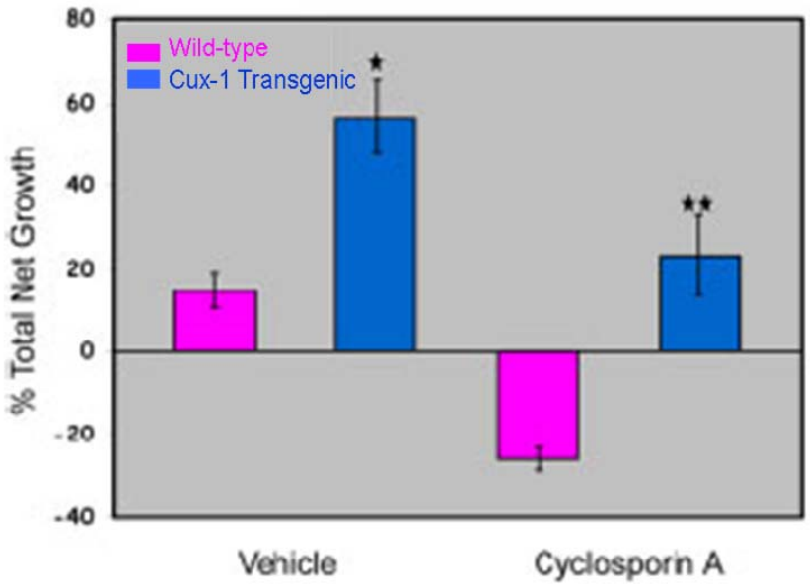


Figure 2-5. Quantification of metanephric kidney growth. Organ culture growth was quantified using the NIH image J program. Net growth per day was determined as the percentage of total area before treatment (baseline) subtracted from total area after treatment collected as a mean of 15 paired wild-type or Cux-1 transgenic metanephroi in six experiments. Net growth per treatment group was determined as the total mean of all percentage growth data during treatment. Results are represented as means ± SEM. The asterisk denotes mean net Cux-1 transgenic vehicle-treated kidney net growth to be significantly ($P < 0.001$) greater than vehicle-treated wild-type net growth. The double asterisk denotes mean cyclosporin A (CsA) -treated Cux-1 transgenic net growth to be significantly ($P < 0.001$) greater than wild-type treated CsA net growth. Image taken from Alcalay et al. (242).

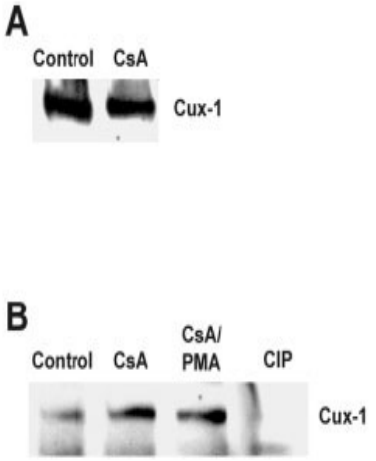


Figure 2-6. Increased phospho-Cux-1 in cyclosporin A (CsA) -treated kidney organ cultures. A: Western blot of total protein lysate from metanephric kidney cultures grown for 48 hr in medium containing either 500 ng/ml CsA or vehicle (control). No change in Cux-1 expression was observed. B: Western blot of phosphorylated protein from metanephric kidney cultures grown for 48 hr in medium containing either 500 ng/ml CsA or vehicle. Phosphoproteins were purified from total kidney organ culture using the QIAGEN PhosphoProtein Purification Kit, according to the manufacturer's directions. Total phosphoproteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane, and phospho-Cux-1 was detected using an anti-Cux-1 antibody. Increased levels of phospho-Cux-1 were observed in CsA-treated kidney cultures (CsA), compared with vehicle-treated kidney cultures (control). Treatment of kidney cultures with CsA and PMA did not result in increased phospho-Cux-1 (CsA/PMA). However, pretreatment with calf intestinal phosphatase resulted in reduced phospho-Cux-1 (CsA/CIP). Results shown are representative of three independent experiments. Image taken from Alcalay et al. (242).

The surface areas of 15 paired wild-type or Cux-1 transgenic metanephroi treated with or without CsA from six separate experiments were compared. The percentage change in mean size of vehicle and CsA-treated metanephroi from wild-type or transgenic animals were paired to control for differences in gestational age. **Figure 2-5** shows that vehicle-treated wild-type metanephroi showed an increase in net percentage growth, while CsA treatment resulted in a decrease in net percentage growth. In contrast, both vehicle- and CsA-treated transgenic metanephroi showed an increase in net percentage growth, although the extent of growth was reduced with CsA treatment (**Figure 2-5**). The differences between percentage net growth of vehicle- and CsA-treated metanephroi were statistically significant.

To determine whether inhibition of calcineurin by cyclosporin changed the phosphorylation state of Cux-1, we evaluated Cux-1 isolated from vehicle- or cyclosporin-treated metanephric kidney cultures. **Figure 2-6A** shows that the levels of total Cux-1 protein were similar between vehicle- or CsA-treated kidney cultures. However, when phosphorylated Cux-1 protein was assessed, we observed a greater than fivefold increase in phosphorylated Cux-1 in the CsA-treated kidney cultures compared with the control cultures (**Figure 2-6B**). Pretreatment of kidneys with both CsA and PMA did not significantly increase the levels of phosphorylated Cux-1 (**Figure 2-6B**). However, no phosphorylated Cux-1 was detected after treatment with calf intestinal phosphatase (**Figure 2-6B**).

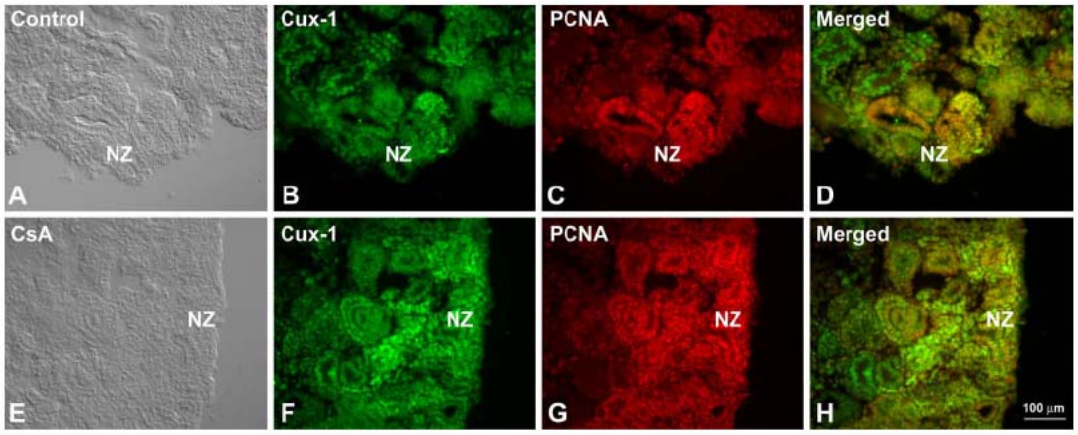


Figure 2-7. Inhibition of cell proliferation by cyclosporin A (CsA) is rescued by ectopic expression of Cux-1. A-H: Sections of kidney organ cultures from Cux-1 transgenic mice treated with vehicle (control, A-D) or CsA (E-H) were labeled with antibodies against Cux-1, to detect both endogenous and ectopic Cux-1 (B,F) and proliferating cell nuclear antigen (PCNA), to detect proliferating cells (C,G). D: Cux-1- and PCNA-positive cells were coexpressed in the vehicle-treated cultures, in regions corresponding to the nephrogenic zone. H: In contrast to CsA-treated wild-type kidney cultures, which showed reduced cell proliferation (see Fig. 2G), CsA-treated Cux-1 transgenic kidney cultures showed high levels of cell proliferation and colocalization of Cux-1 and PCNA. NZ, nephrogenic zone. Scale bar = 100 µm. Image taken from Alcalay et al. (242).

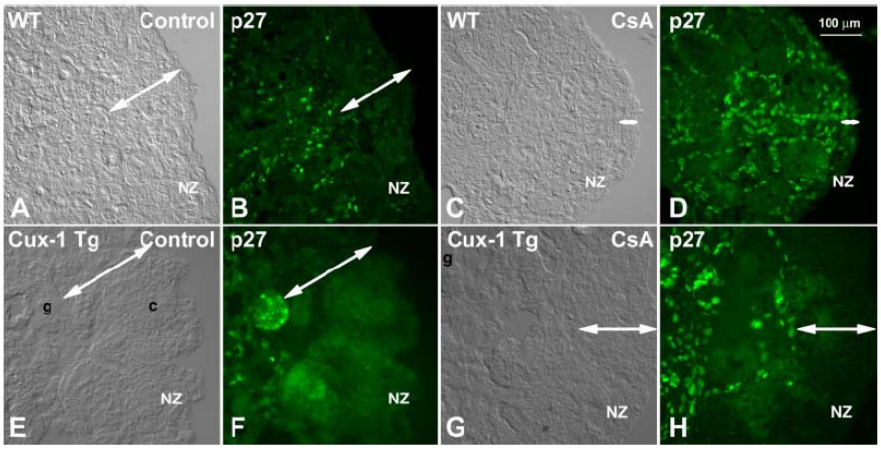


Figure 2-8. The nephrogenic zone is rescued in Cux-1 transgenic kidney organ cultures treated with cyclosporin A (CsA). A-H: Sections of wild-type (A-D) and Cux-1 transgenic (E-H) kidney organ cultures were labeled with antibodies directed against p27. p27 is normally not expressed in the nephrogenic zone, but is up-regulated in maturing glomeruli and tubules, where it is associated with cell cycle arrest and terminal differentiation. B,F: p27 expression was absent from the nephrogenic zone of vehicle (control) -treated wild-type (B) and Cux-1 transgenic (F) kidney organ cultures. D: p27 was ectopically expressed at the periphery of the CsA-treated wild-type kidney organ cultures, similar to CnA-α -/- kidneys (172). H: In contrast, p27 expression was absent from the nephrogenic zone of CsA-treated Cux-1 transgenic kidney organ cultures. Arrows indicate the distance between the edge of the kidney and the outermost p27 expressing cells. NZ, nephrogenic zone; g, glomerulus; c, comma-shaped body. Scale bar = 100 μm. Image taken from Alcalay et al. (242).

Figure 2-9. A potential model of Cux-1 regulation by calcineurin. Our previous results show that Cux-1 down-regulates the expression of the cyclin kinase inhibitor p27 (20). Because p27 induces cell cycle arrest, increased Cux-1 expression or activity would result in increased cell cycle progression. Our present results show that inhibition of the phosphatase calcineurin by cyclosporin A (CsA) results in increased phospho-Cux-1, which is unable to bind DNA (55, 59), and suggests that Cux-1 activity may be regulated by calcineurin. In addition to down-regulation ofp27 by Cux-1, CsA treatment may also result in p27 activation independent of Cux-1. Image taken from Alcalay et al. (242).

To determine the basis for the rescued growth of Cux-1 transgenic metanephroi in the presence of CsA, we examined Cux-1 expression and cell proliferation in vehicle- or CsA-treated transgenic kidney cultures. Figure 7 shows that, similar to wild-type, Cux-1 was expressed throughout the nephrogenic zone in both vehicle- and CsA-treated metanephroi (**Figure 2-7B,F**). However, in contrast to wild-type kidney cultures, PCNA was not down-regulated in CsA-treated Cux-1 transgenic metanphroi, but was colocalized with Cux-1 (**Figure 2- 7G,H**). We next examined p27 expression in vehicle- and CsA-treated wild-type and Cux-1 transgenic metanephroi. p27 is normally expressed in maturing glomeruli and tubules, after the down-regulation of Cux-1. While p27 was absent from the nephrogenic zone of vehicle-treated kidneys (**Figure 2-8B,F**), p27 was ectopically expressed in the nephrogenic zone of CsA-treated wild-type kidney organ cultures (**Figure 2-8D**), similar to CnA α(-/-) kidneys. In contrast, p27 was not expressed in the nephrogenic zone of the CsA-treated Cux-1 transgenic kidney organ cultures (**Figure 2-8H**).

Discussion

Cux-1 is a murine homeobox gene that is related to the Drosophila cut gene. Mammalian Cut protein expression appears to be restricted to proliferating cells in many different tissues (13) where they function as cell cycle-dependent transcriptional repressors. Targets of Cux-1 repression include the cyclin kinase

inhibitors p21 and p27 (59, 60).

During nephrogenesis, maturing nephrons proceed through an orderly sequence of developmental stages that can be distinguished morphologically. These stages are renal vesicle (stage I), comma- and S-shaped body (stage II), developing capillary loop (stage III), and maturing glomerulus (stage IV). The developing kidney displays a spatial gradient of differentiation in which nephrons at the earliest stages of development (stage I and II) are restricted to the nephrogenic zone immediately beneath the renal capsule, while progressively more mature nephrons (stage III and IV) are located toward the center of the kidney (177). We have previously shown that Cux-1 is highly expressed in the nephrogenic zone of developing kidneys, where it is associated with cell proliferation (19). In addition, transgenic mice ectopically expressing Cux-1 in the developing kidney exhibit renal hyperplasia, resulting from aberrant repression of p27 in maturing glomeruli and tubules.

Previous studies have demonstrated that loss of CnA α results in disruption of the cell cycle in kidney development (172). The nephrogenic zone of developing kidneys isolated from CnA α(-/-) mice showed decreased proliferation and increased cell death that was associated with increased levels of p27. In addition, glomeruli present in the CnA α(-/-) mice were devoid of mesangial cells. In contrast, Cux-1 transgenic mice exhibit mesangial cell hyperplasia. Moreover, mesangial cells isolated from Cux-1 transgenic mice are not growth restricted in the absence of serum, but progress through the cell cycle (70).

To determine whether the decreased cell proliferation in kidneys from CnA α (-/-) mice resulted from the down-regulation of Cux-1, we examined Cux-1 expression in CnA α(-/-) kidneys and in kidney cultures treated with the antibiotic CsA. CsA specifically inhibits calcineurin, and previous studies have shown that administration of CsA to pregnant rabbits during the period when nephrogenesis is initiated results in defects in the nephrogenic zone (176). Both in CnA α(-/-) kidneys and in kidneys treated with CsA, our results showed that Cux-1 was expressed throughout the nephrogenic zone (**Figure 2-1**), similar to the expression in wild-type or vehicle-treated kidneys. However, in contrast to wild-type or vehicle-treated kidneys, Cux-1 expression was not associated with cell proliferation in CnA α(-/-) kidneys(**Figure 2-1**) or in kidneys treated with CsA(**Figure 2-2**). Moreover, kidney cultures treated with CsA showed increased levels of phosphorylated Cux-1(**Figure 2-6**), compared with vehicle-treated kidneys, although the total amount of Cux-1 was not different between CsA and vehicle-treated kidneys. Because the ability of Cux-1 to bind DNA is negatively regulated by phosphorylation, these results suggest that Cux-1 activity may be regulated by calcineurin. The loss of calcineurin, either in the CnA α(-/-) mice or by pharmacologic inhibition, would then result in the maintenance of Cux-1 in an inactive state. A potential model of Cux-1 regulation by calcineurin is shown in **Figure 2-9**.

To determine whether increased Cux-1 expression could overcome the loss of calcineurin, we cultured kidneys isolated from Cux-1 transgenic mice with cyclosporin. In contrast to wild-type kidneys, the transgenic kidneys were not growth

inhibited, but continued to increase in size(**Figure 2-5**). Moreover, CsA-treated Cux-1 transgenic kidneys showed extensive cell proliferation in the nephrogenic zone, with reduced expression of p27 (**Figure 2-8**). We have previously shown that Cux-1 expression in kidneys isolated from Cux-1 transgenic mice is significantly elevated when compared with wild-type mice (20). One possibility is that the amount of Cux-1 protein produced by the transgenic kidneys exceeds the rate of phosphorylation, resulting in significant levels of active Cux-1 protein that is able to bind DNA and repress target genes, including p27. Taken together, these results demonstrate that the ectopic expression of Cux-1 rescued the cell proliferation defects in the nephrogenic zone induced by cyclosporin, and suggest that Cux-1 may be regulated by calcineurin during kidney development *in vivo*.

CHAPTER THREE

MUTATION IN CUX-1 MODIFIES THE MURINE POLYCYSTIC KIDNEY DISEASE PHENOTYPE

Abstract

The murine transcription factor Cux-1 is structurally related to Drosophila *cut* and contains 4 DNA binding domains (3 cut repeats, 1 homeodomain). Cux-1 is highly expressed in cells undergoing proliferation during kidney development Polycystic kidney disease (PKD) is characterized by increased mitotic activity and dedifferentiation of tubular epithelia, leading to cyst formation. Previously, we observed ectopic Cux-1 expression in *cpk* and *Pkd-1* null kidneys, two murine models of PKD. Since Cux-1 might be involved in the PKD pathway, it is possible that mutation in *cux-1* modifies cystic disease. To test this, we crossed mice carrying a deletion of the first cut repeat (*cux-1ΔCR1*) with *cpk* cystic mice to generate mice homozygote for both genes (termed *cux-1ΔCR1/cpk*). Cystic kidneys were collected from *cux-1ΔCR1/cpk* mice at post-natal day 10 with kidney weight measured as percent body weight. Cystic kidneys were sectioned and labeled for Cux-1 and PCNA, a marker for cell proliferation. In addition, blood serum was collected to determine blood urea nitrogen levels (BUN), an indicator of renal function. Results demonstrated that *cux-1ΔCR1/cpk* cystic kidneys were significantly larger than *cpk* kidneys. Moreover, *cux-1ΔCR1/cpk* mice had higher BUN levels than *cpk* indicating more severe renal failure. Taken together, these results suggest that the *cux-1ΔCR1* gene modifies *cpk* cystic disease.

Introduction

Polycystic kidney disease (PKD) is a term applied to a group of inherited disorders characterized by the presence of renal cysts, however, multiple organs are usually affected. Human autosomal dominant polycystic kidney disease (ADPKD) results from mutations in one of two genes, PKD1 or PKD2 that encode polycystin-1 and Polycystin-2 proteins, respectively (178-181). Human autosomal recessive polycystic kidney disease (ARPKD) results from mutations within a single gene, PKHD1 (polycystic kidney and hepatic disease 1), encoding fibrocystin/polyductin (182-183). Proteins that are mutated in human PKD (Polycystin-1, Polycystin-2, fibrocystin/polyductin), and in animal models of PKD (Cystin, Polaris), co-localize to the primary cilia (110, 184-186). This suggests that mutations in ciliary proteins affect common or overlapping signaling pathways resulting in PKD (187). The process of cyst formation in PKD is thought to involve various mechanisms including cell proliferation, fluid secretion, dedifferentiation, abnormal basement membrane formation, matrix remodeling, apoptosis, and alteration in cellular polarity (98, 188, 189). Growing evidence suggests that PKD is a developmental disorder (101, 138, 148, 184, 190-193).

The *cpk* gene, when crossed onto different genetic backgrounds (CD1, Balb/c, DBA/2J, CAST/Ei) results in an altered phenotype (pancreatic and biliary ductal cysts) compared to the B6 background from which it originated (103, 104, 108, 194). The authors concluded that phenotypic differences between strains may be attributed to the presence of modifying genes. Phenotype modification is

identified when extreme phenotypes are observed (**Figure 1-16**). In the case of PKD, modification is clear if the disease is ameliorated or exacerbated. For example, cystic kidney weight is used as a phenotypic marker to indicate PKD severity (103, 104, 120, 121). The *wa-2* point mutation (valine→glycine at position 743) in EGFR decreases tyrosine kinase activity (133). Richards et al. (131) demonstrated that *wa-2* improves kidney function associated with decreased cyst formation in *orpkd.* The authors found the *wa-2* mutation is most potent in reducing cysts in collecting ducts where EGFR is mislocalized to the apical surface.

The *wa-2* mice carry the same phenotype as *cux-1ΔCR1* in wavy hair, curly whiskers, with a lactation defect occurring in females. Mutant *cux-1ΔCR1* mice do not develop PKD and have normal kidney (37). Cux-1 protein is ectopically expressed in *PKD1* knockout and *cpk* cystic kidneys suggesting that it may be involved in the PKD pathway (19, 135). From this observation *cux-1ΔCR1* could be considered as a candidate modifying gene for PKD. In the present study a reverse genetics strategy was approached to generate *cpk* mice carrying a hypomorphic allele of *cux-1* to determine if mutation within *cux-1* affects the progression of disease.

Materials and Methods

Animals

To determine if the *cux-1ΔCR1* gene is a candidate modifier gene of PKD we decided to use a reverse genetics strategy. As an initial step, the *cux-1ΔCR1* gene was crossed onto mice carrying the *cpk* gene (*cpk*/+) to generate homozygotes for

both *cux-1ΔCR1* and *cpk* (termed *cux-1ΔCR1/cpk*) present on a mixed B6/129S4 background. *cpk* /+ and *Cux-1ΔCR1 /+* mice were purchased from Jackson Laboratory (Bar Harbor, ME), and stock colonies are maintained at the University of Kansas Medical Center. Identification of +/+, *cpk* /+, and *cpk* genotypes was determined by PCR, as previously described (184). The presence of the *Cux-1ΔCR1* mutation was identified by Southern blot analysis, as described (37). Males homozygous for *Cux-1ΔCR1* and heterozygous for *cpk* were crossed with female mice heterozygous for both *cux-1ΔCR1* and *cpk* to generate double homozygote *cux-1ΔCR1/cpk* mice (**Figure 3-1**). All *Cux-1ΔCR1*, *cpk*, and *cux-1ΔCR1/cpk* mice analyzed were on the same B6129SF1/J mixed genetic background. All protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. The University of Kansas Medical Center is fully accredited by the American Association of the Accreditation of Laboratory Animal Care.

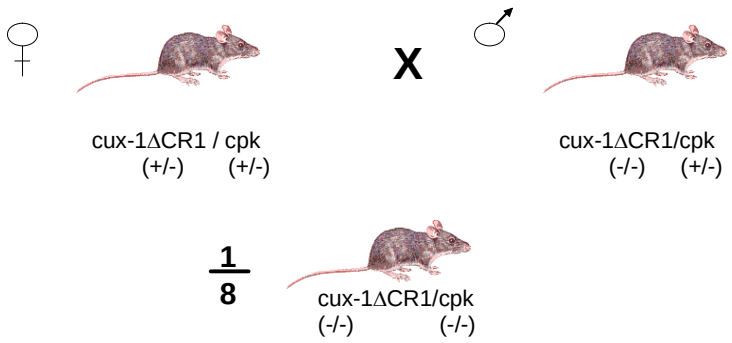


Figure 3-1. Breeding strategy to generate cux-1ΔCR1/cpk double homozygote animals.

Kidneys were harvested from 10 day old (P10) *cux-1ΔCR1/cpk* mice because this is the earliest time-point where cystic kidneys can be distinguished from non-cystic kidneys. Kidney weight was then standardized as a percentage of total body weight.

Characterization of cystic phenotype

Cystic kidneys were collected from 3-day-old and 10-day-old cpk and *Cux-1ΔCR1/cpk* mice. Kidney length/Crown rump measrurements (see **Figure 3-2**) were collected. Animal body weight (BW) was measured in grams and converted to milligrams followed by the collection and weight measurement of bilateral kidneys in milligrams. Total cystic kidney weight (KW) was divided by body weight and calculated to determine KW as a percent BW. Kidney length measurements of bilateral kidneys were performed and divided by crown-rump measured in centimeters (cm). Cystic kidney mid-sagittal sections (5μm) were utilized to stage cysts, as described previously (195). Cysts were considered early stage if there were less than 50 cyst-lining epithelial cells, intermediate if there were 51-200 cyst-lining epithelial cells, and advanced stage if greater than 200 cyst-lining cells.

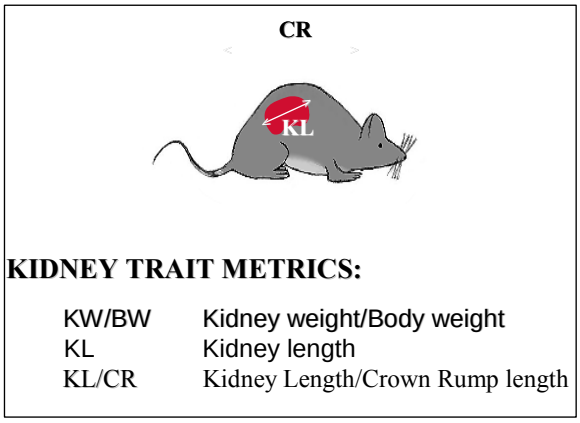


Figure 3-2. Characterization of kidney size by established quantitative measurements.

Protein analysis

Whole cell lysates (30μg) and nuclear extracts (20μg) of Human epidermal carcinoma cells (A-431) were purchased from Santa Cruz and loaded on 4-15% SDS-PAGE gels. Standard western blot protocol was performed with PVDF membranes being subjected to Cux-1 (Santa Cruz), p27 (Santa Cruz) and EGFR (Cell Signaling). β-actin (sigma) and ponceau-S were used to verify equivalent protein loading. In an additional experiment whole cell lysates from *Cux-1ΔCR1* kidneys were also analyzed for EGFR protein expression.

Serum chemistry

Blood was collected by intra-cardiac puncture and immediately centrifuged at 2000Xg for serum collection. Blood urea nitrogen (BUN) was determined using an autoanalyzer (Physicians Reference Laboratory, LLC, Overland Park, KS, USA).

Experimental Animals

cpk and *cux-1ΔCR1* mice were obtained from Jackson Laboratories with stock colonies maintained at the University of Kansas Medical Center animal facilities. The Cux-1ΔCR1 gene present on the B6/129 strain was crossed three times onto the B6 background. Males homozygous for *cux-1Δ*CR1 and heterozygous for cpk were genotyped as previously described[2], and crossed with female mice heterozygous for both *cux-1Δ*CR1 and *cpk* to generate double

homozygote cux-1ΔCR1/cpk mice. All protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. The University of Kansas Medical Center is fully accredited by the American Association of the Accreditation of Laboratory Animal Care.

Characterization of cystic phenotype

Cystic kidneys were collected from 10 day-old *cpk* kidneys. Animal body weight (BW) was measured in grams and converted to milligrams followed by the collection and weight measurement of bilateral kidneys in milligrams. Total cystic kidney weight (KW) was divided by body weight and calculated to determine KW as a percent BW. Kidney length measurements of bilateral kidneys were performed and divided by crown-rump measured in centimeters (cm).

Blood was collected by intra-cardiac puncture and immediately centrifuged at 2000Xg for serum collection. Blood urea nitrogen (BUN) analysis was performed by Physicians Reference Laboratory; Overland Park, KS.

Cystic kidney mid-sagittal sections (5μm) stained with hematoxylin and eosin were utilized to stage cysts as described previously (195). Cysts were considered early stage if there were less than 50 cyst-lining epithelial cells, intermediate if there were 51-200 cyst-lining epithelial cells, and advanced stage if greater than 200 cyst-lining cells.

Dolichos Biflorus Agglutinin (Vector) labeling and Lotus Tetraglobin Lectin (Vector) were used to label for collecting duct- and proximal tubule-derived cells

respectively. All whole kidney images were taken using a "camera" while histological pictures were performed by utilizing a Leica optronics® microscope and DMR camera system.

Statistics

In all studies, a one-way ANOVA was performed. If significance between the genotypes existed ($P \leq 0.05$) post-hoc analysis by least significant difference (LSD) was performed to determine statistical significance ($p \leq 0.05$) between groups. All statistical analyses were performed using the Statview® statistical program.

Results

Reduced EGFR tyrosine kinase activity has been implicated in the progression of PKD particularly in the *cpk* and *orpk* models of the disease. A point mutation in EGFR (termed the *wa-2* mutation) results in the reduction of EGFR tyrosine kinase activity with animals carrying an identical phenotype to that of mutant Cux-1 mice (termed *cux-1ΔCR1*) which carry a 246 amino acid deletion of Cux-1. Therefore, it is possible that Cux-1 is involved in the EGFR pathway.

Preliminary results determined that there were normal amounts of EGFR in *cux-1ΔCR1* kidneys (**Figure 3-3A**). To determine if Cux-1 is involved in the EGFR pathway, human epidermal carcinoma cells (A-431 cells) were treated with epidermal growth factor (EGF) ligand. Our results showed that stimulation with EGF attenuated Cux-1 protein levels when EGFR is upregulated (**Figure 3-3B,C**). Cux-1 expression is attenuated in EGF stimulated cells (**Figure 3-3c**) is supported by

reports showing EGF stimulation inhibits cell proliferation in A-431 cells (196, 197). Since Cux-1 is involved in p27 expression in renal systems we looked at p27 levels within EGF treated epidermal carcinoma cells and found that p27 protein was also upregulated (**Figure 3-3D**).

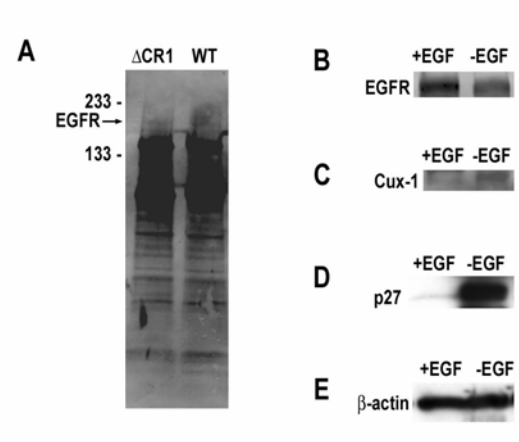
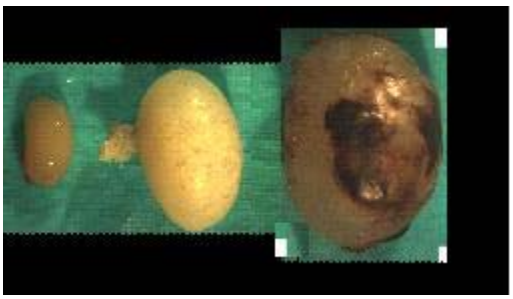


Figure 3-3. Epidermal Growth Factor Receptor (EGFR) regulation of Cux-1 expression. (A) Similar levels of the 175KD EGFR protein were detected in 2-month *cux-1ΔCR1* (ΔCR1) and wild-type (WT) kidney lysates. Stimulation of human epidermal carcinoma cells (A431) with 5ng/ml EFG ligand resulted in (B) the upregulation of EGFR, (C) downregulation of Cux-1 and (D) the cyclin kinase inhibitor p27. (E) Equivalent levels of β-actin protein confirm equal loading of nuclear lysates (C, D).

In previous studies, our laboratory demonstrated that Cux-1 is ectopically expressed in cystic cells during the later stages of *cpk* cystic disease and during embryogenesis of *PKD1* homozygote knockout mice (19, 135). Mutation in these cyst producing genes results in the ectopic expression of Cux-1. This suggests the involvement of Cux-1 in PKD pathways. If Cux-1 is involved in the PKD pathway, it is possible that mutation in *cux-1* modifies cystic disease. In addition, since the *wa-2* mutation within EGFR carries a similar phenotype to *cux-1ΔCR1* (37, 133), it prompted us to predict that *cux-1ΔCR1* would attenuate cyst growth similar to that observed in *wa-2/orpkd* cystic kidneys (131).

We crossed *cux-1ΔCR1* mice with *cpk* mice to generate mice homozygous for both genes (*cux-1ΔCR1/cpk*). The *cux-1ΔCR1* gene, present on a 129J genetic background, was backcrossed onto the B6 background where *cpk* is present, resulting in a 75% B6 25% 129J mixed genetic background. Mice carrying the Cux-1ΔCR1 mutation have curly whiskers, wavy hair, and lactation defects (37), but have normal kidneys (see Chapter 1). Surprisingly, when this mutation was combined with the *cpk* mutation, the double homozygous (*cux-1ΔCR1/cpk*) mice developed cystic kidneys that were significantly larger than the cystic kidneys from mice carrying only the mutation in *cpk* at post-natal day 17 (**Figure 2-4**). This qualitatively suggested that the *cux-1ΔCR1* gene accelerated polycystic kidney disease (PKD).



Wild-type *cpk* *cux-1ΔCR1/cpk*

Figure 3-4. Large Cux-1ΔCR1/cpk kidneys were observed at post-natal day 17.

PKD disease severity has been linked to and directly correlated with kidney size. Larger cystic kidneys represent a more severe stage of the disease whereas smaller sized kidneys represent mild PKD. Mrug et al. demonstrated this correlation when quantifying kidney size by kidney length (KL), kidney length/ crown-rump (KL/CR) and kidney weight taken as a percentage of body weight (KW/BW). They

quantified *cpk* kidney size from well over 400 different animals using these parameters at post-natal day 10 and found that kidney size is consistent. Therefore, any alteration in the renal cystic KW/BW would represent a modification of the disease.

Bilateral kidneys were harvested at post-natal day 10 (P10) and KW and BW measurements were collected to calculate KW standardized as a percentage of BW, to quantify renal cystic disease severity (16,33). By gross appearance, kidneys from *cux-1ΔCR1/cpk* mice were significantly larger than kidneys from *cpk* mice on the same mixed B6/129 background or pure B6 alone (**Figure 3-5A,B**). *cux-1ΔCR1/cpk* cystic kidneys were significantly larger then *cpk* kidneys regardless if *cpk* was present on B6/129 or B6 backgrounds. In contrast to the effect of the presence of *cux-1ΔCR1 homozygosity with cpk*, partial gene dosage of *cux-1ΔCR1* did not increase cystic kidney size. The presence of cpk on the mixed B6/129 background resulted in an increase in kidney size compared to cpk on B6. The increase in kidney size apparently occurs early in development as *cux-1ΔCR1/cpk* kidneys were significantly larger then *cpk* kidneys both at post-natal days 3 and 10. (Fig. 5A, B). KL and KL/CR levels were elevated but did not reach statistical significance ($p \leq 0.05$).

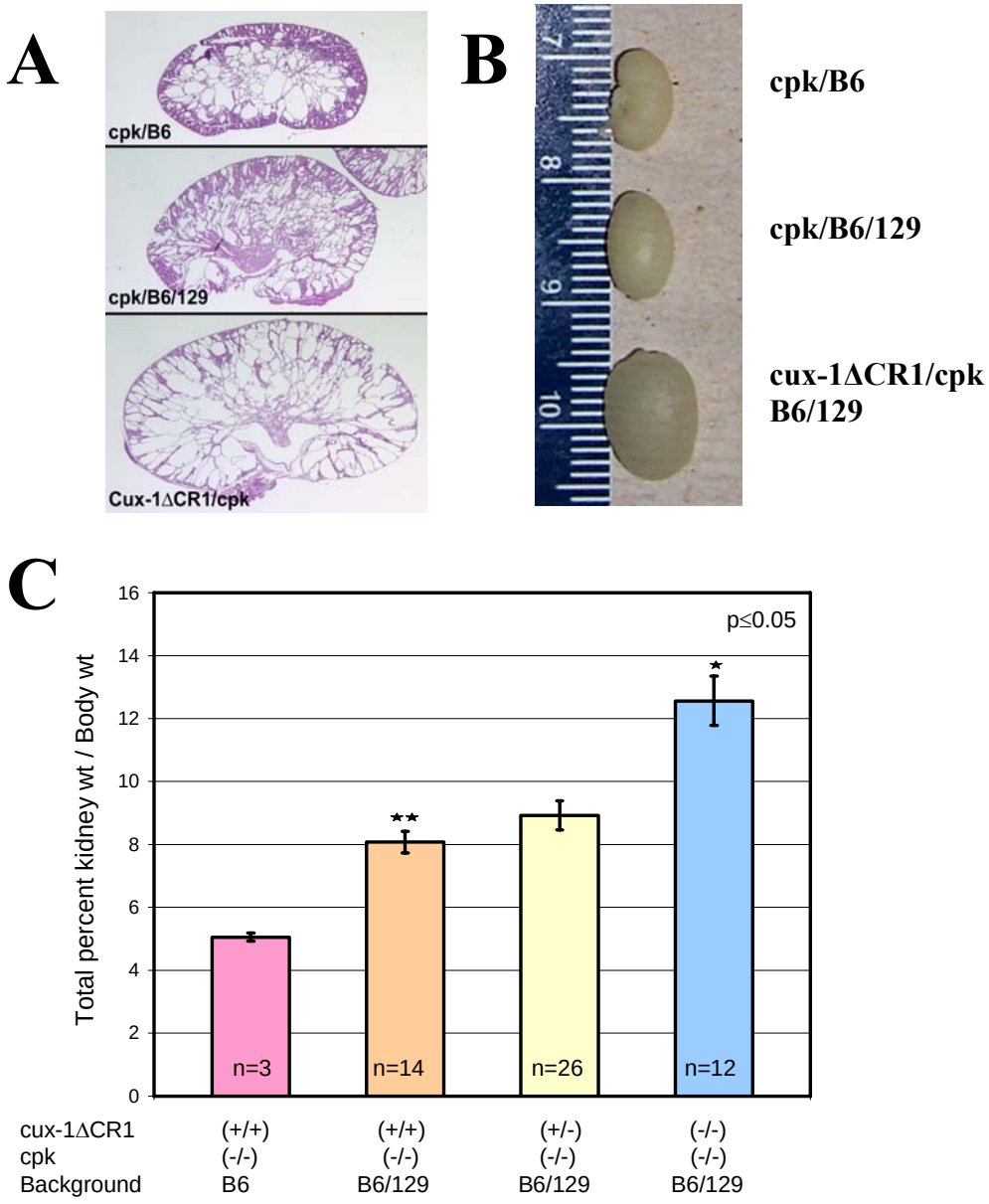


Figure 3-5. Cux-1ΔCR1/cpk kidneys are significantly larger compared to cpk. (A) Hematoxylin and eosin staining of midsagittal cystic kidney sections showing the appearance of larger cysts with *cux-1ΔCR1/cpk* kidneys compared to cpk. (B) Gross appearance of kidney size depicting *cux-1ΔCR1/cpk* kidneys to be larger then those of cpk. (C) KW/BW analysis revealed significantly larger Cux-1ΔCR1/cpk kidneys compared to cpk. (*) Denotes statistical significance determined by ANOVA and LSD post-hoc analysis (p≤0.05).

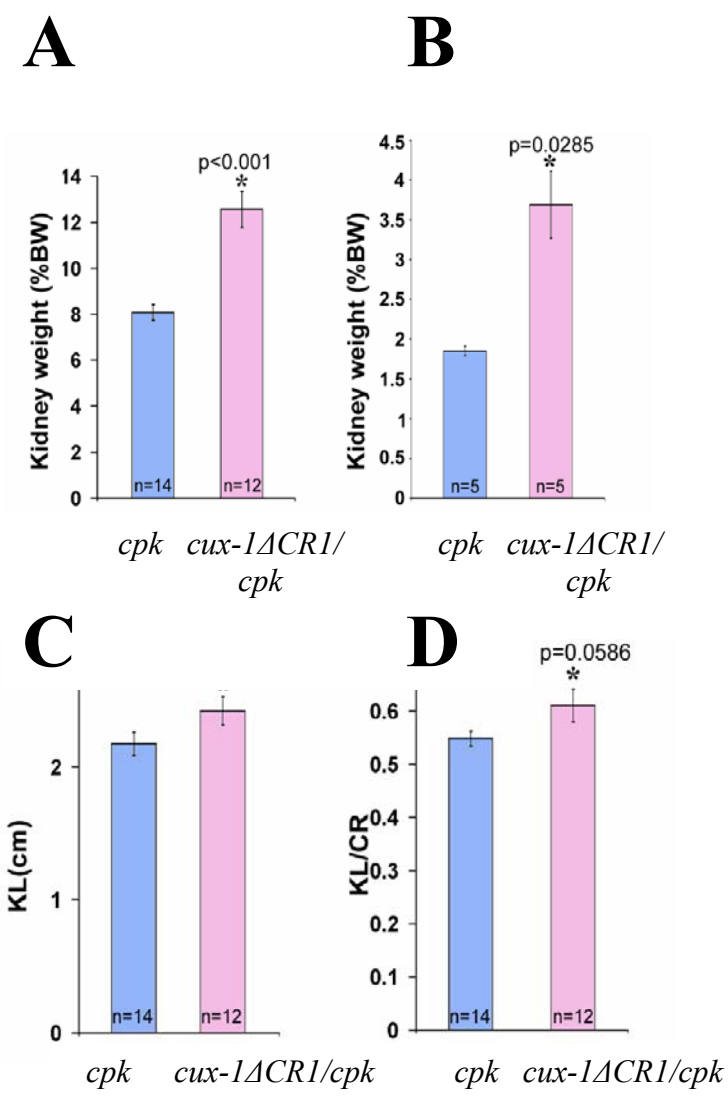


Figure 3-6. Quantitative kidney size measurements in cpk kidneys. (A Kidney weight (KW) standardized as a percentage of Body Weight (BW) represented as mean values ±SEM. (*) Denotes that kidneys from *cux-1ΔCR1/cpk* 10-day-old mice were significantly larger (P≤0.0001) than kidneys from *cpk* 10-day-old mice in, and (B) that kidneys from *cux-1ΔCR1/cpk* 3-day-old mice were significantly larger (P≤0.05) than kidneys from *cpk* 3-day-old mice. (C) KL, and (D) KL/CR demonstrated modestly increased size. (*) Denotes statistical significance determined by ANOVA and LSD post-hoc analysis (p≤0.05).

Reduced renal function and increased kidney size correlate with the progression of PKD. Many studies have demonstrated that large cystic kidneys are directly correlated with reduced renal function (103, 104, 120, 121). Blood urea nitrogen (BUN) and blood Creatinine (CR) are physiological markers used for the quantitative analysis of renal function. To determine if the larger cystic kidneys observed in *cux-1ΔCR1/cpk* mice results in reduced renal function, BUN and CR levels were assessed. BUN and CR levels were elevated in *cux-1ΔCR1/cpk* mice (Figure 3-6) demonstrating that in addition to having significantly large cystic kidneys *cux-1ΔCR1/cpk* mice have reduced renal function.

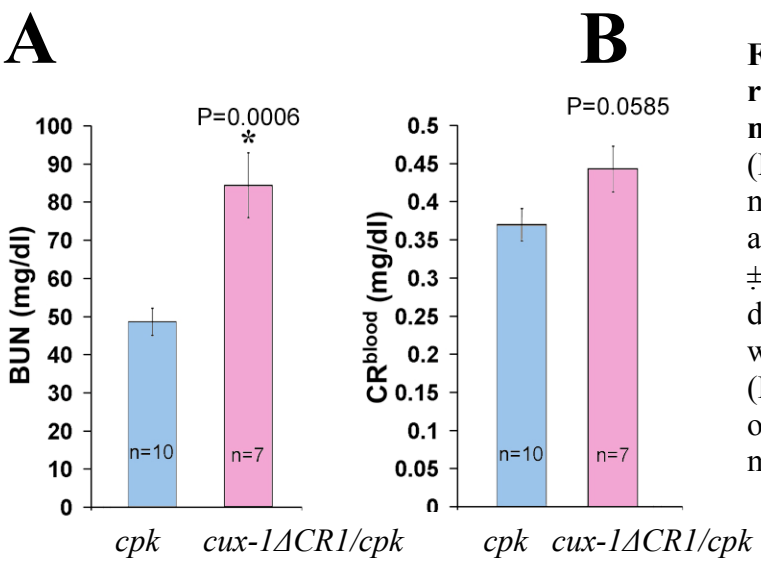


Figure 3-7. Renal function is reduced in Cux-1ΔCR1/cpk mice. (A) Blood Urea Nitrogen (BUN) and (B) Creatinine (CR) measurements were collected and represented as mean values ±SEM. (*) Denotes BUN in 10-day-old *Cux-1ΔCR1/cpk* mice were significantly greater (P≤0.001) than BUN in 10-day-old *cpk* mice. A modest rise in mean blood CR was observed.

Large cystic kidneys observed in *cux-1ΔCR1/cpk* mice may develop as a result of increased numbers of cysts, increased cyst size or both an increase in the number and size of cysts. To further analyze the *cux-1ΔCR1/cpk* phenotype, mid-saggital sections of cystic kidneys were stained with Haematoxylin and Eosin. There

cysts in *cux-1ΔCR1/cpk* kidneys appeared to be larger compared to *cpk* kidneys (**Figure 3-6**). When cysts were counted from midsaggital sections of *cpk* kidneys (**Figure 3-7A, B**). Cysts were then counted to determine if *cux-1ΔCR1/cpk* kidneys have a greater number than *cpk* kidneys. Histological analysis showed that the numbers of cysts between *cux-1ΔCR1/cpk* and *cpk* kidneys were statistically similar. There even appeared to be relatively reduced numbers of cysts in *cux-1ΔCR1/cpk* kidneys. Since cysts from *cux-1ΔCR1/cpk* kidneys appeared larger or more developed then cysts from *cpk* kidneys (**Figure 3-4A and Figure 3-7A**) we decided to quantify cysts morphology.

Although the overall number of renal cysts was not different between *cpk* and *cux-1ΔCR1/cpk* mice (**Figure 3-7b**) the size of the cysts appeared to be increased in the kidneys from *cux-1ΔCR1/cpk* mice. Cyst numbers were quantified in regard to the developmental stage of each individual cyst. The developmental stage of the renal cysts in *cux-1ΔCR1/cpk* and *cpk* mice was determined by counting the number of cells lining the cysts (see Chapter 3 materials and methods). The numbers of intermediate stage of development cysts between *cux-1ΔCR1/cpk* and *cpk* were statistically similar. However, the results showed that kidneys from *cux-1ΔCR1/cpk* mice had increased numbers of cysts in a more advanced developmental stage in comparison to age-matched kidneys from *cpk* mice (**Figure 3-7c**). This was associated with a reduced numbers of early stage cysts in the kidneys from

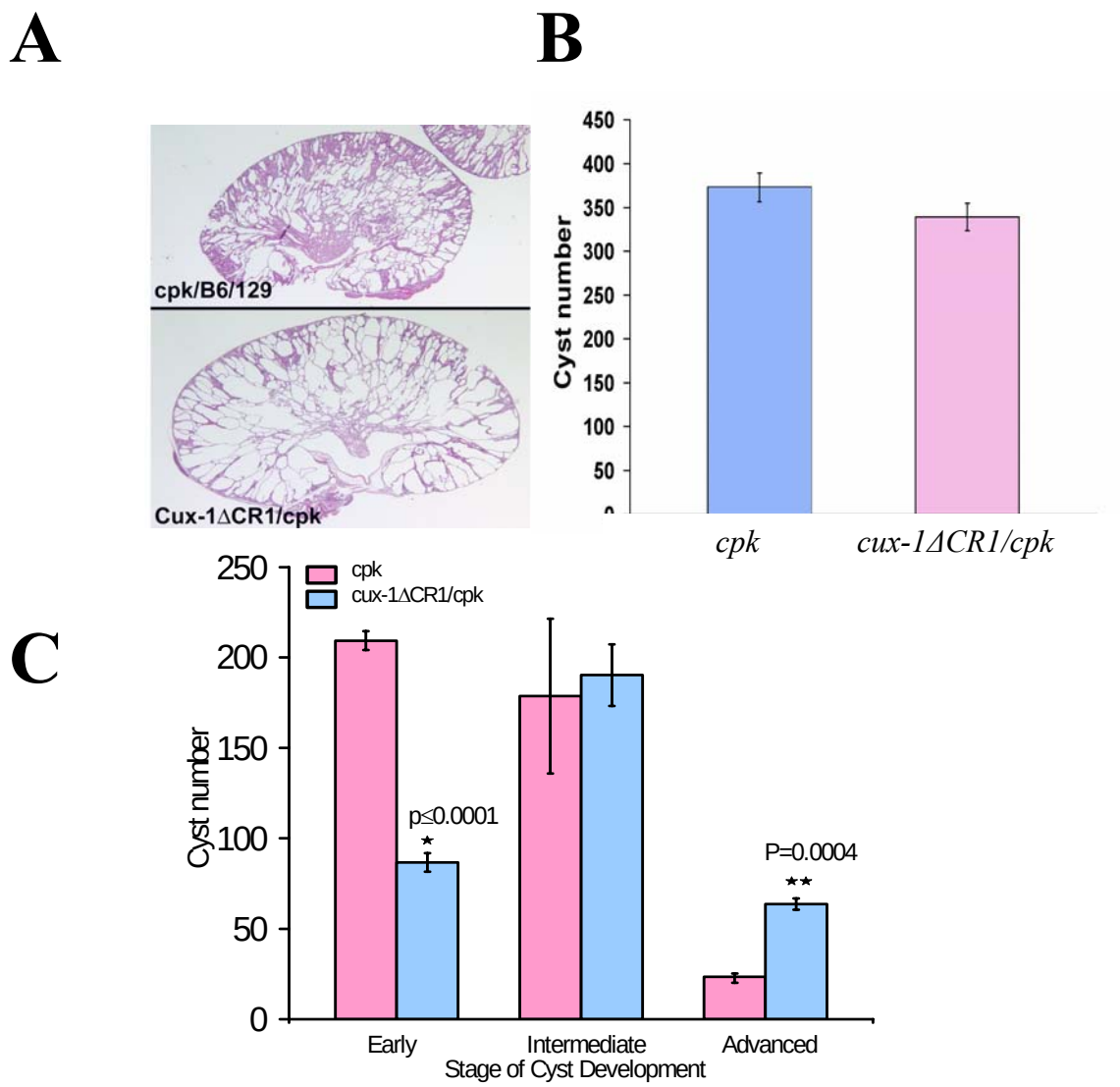


Figure 3-8. Characterization of *cux-1ΔCR1/cpk* cystic kidneys. a, b: Representative midsagittal sections of cystic kidneys isolate from 10-day-old *cpk* (a) and *cux-1ΔCR1/cpk* (b) mice. c. The overall number of cysts is unchanged in mid sagittal sections of kidneys from 10-day-old *cpk* and *cux-1ΔCR1/cpk* mice. d: Numbers of cysts present in Early, Intermediate and Advanced stage of development. Cysts were considered early stage if there were less than 50 cyst-lining epithelial cells, intermediate if there were 51-200 cyst-lining epithelial cells, and advanced stage if greater than 200 cyst-lining cells. (*) Denotes there are significantly less early forming cysts in *cux-1ΔCR1/cpk* and (**) denotes significantly more advanced stage well developed cysts. Each bar represents the mean of three different animals of the indicated genotype ±SEM. e-h. *cux-1ΔCR1/cpk* cysts are predominately derived from collecting-duct origin.

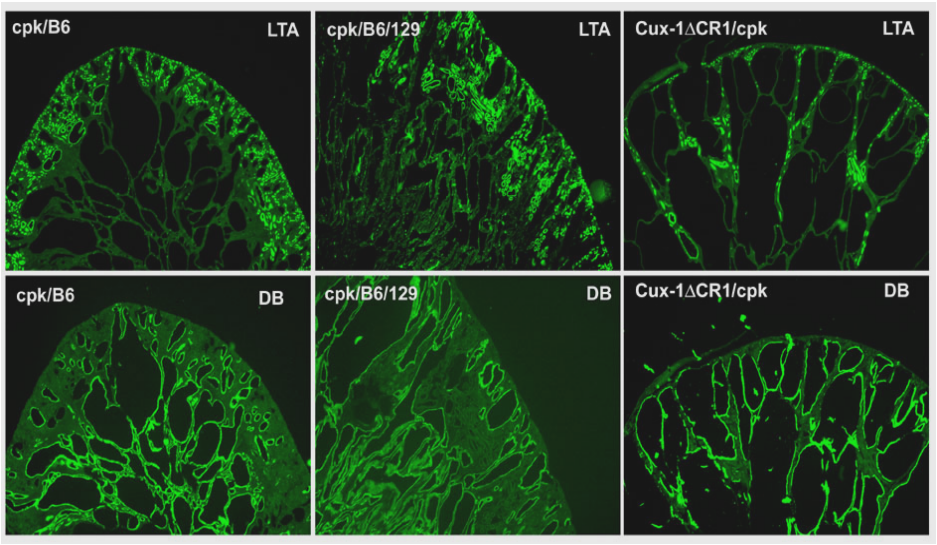


Figure 3-9. Cyst derivation in *cpk* and *cux-1ΔCR1/cpk* kidneys is similar. Mid-sagittal sections of kidneys from *cpk* (e and g) and *cux-1ΔCR1/cpk* (f and h) mice were labeled for lotus tetragonolobus agglutinin (LTA) to identify proximal tubules (e and f) or dolichus bifluorus agglutinin DBA to identify collecting ducts (g and h). Bar, 200μm.

cux-1ΔCR1/cpk mice compared to kidneys from age-matched *cpk* mice. These results support the idea that *cux-1ΔCR1* accelerates PKD cyst progression.

Cystogenesis occurs during two stages in *cpk* kidneys. Proximal tubules develop small cystic dilations during the late fetal stage continuing through the first neonatal week of life. However the larger definitive cysts, exclusively derived from the collecting ducts, occur in the later stages of the disease. Cux-1 is expressed at embryonic day 13.5 (E13.5) prior to the observation of proximal tubule cystic dilations initiating at E16.5. Since *cux-1ΔCR1* carries the same promoter as wild-type *cux-1* (37) and high levels of Cux-1 correlating with cell proliferation occur during early nephrogenesis (19), it is plausible that proximal tubule derived cysts are

more abundant than collecting duct cysts in *cux-1ΔCR1/cpk* kidneys. Another possibility for the larger *cux-1ΔCR1/cpk* cystic kidneys is that there are more proximal tubule-derived cysts in addition to the typical number of collecting duct cysts. To begin to address these possibilities, Dolichos Biflorus Agglutinin (DBA) labeling and Lotus Tetraglobin Lectin (LTL) were used to label for collecting duct- and proximal tubule-derived cells respectively. The pattern of cystogenesis characteristic of the *cpk* mutation was unchanged within *cux-1ΔCR1/cpk* kidneys on the mixed B6/129 background (**Figure 3-4A** and **Figure 3-7A**). Cysts were found both within the outer cortex and interior medulla. However, when the *cpk* gene is present on the B6 background cysts are primarily located in the interior renal medulla (**Figure 3-4A**). *cpk* mice undergo two phases of cystic disease (104, 198). Initially, *cpk* mice develop proximal tubule cysts that resolve by postnatal day 7. This is followed by a second phase of cystogenesis, in which cysts develop primarily from collecting ducts, resulting in massive enlargement of the kidneys. To determine if cyst derivation is altered in *cux-1ΔCR1/cpk* LTA and DBA labeling were performed (See Chapter 3 Materials and Methods) to examine the presence of proximal-tubule and collecting-duct cysts respectively. In kidneys from both *cux-1ΔCR1/cpk* and *cpk* mice, the cysts were primarily restricted to the collecting ducts, with few proximal tubule cysts, although the collecting duct cysts in the *cux-1ΔCR1/cpk* kidneys were larger than those in the cpk kidneys (**Figure 3-8**).

Discussion

The results from this chapter demonstrate that mutation in Cux-1 can alter PKD. We crossed the *cux-1ΔCR1* gene onto *cpk* mice and observed modification of the PKD phenotype. The *cux-1ΔCR1* gene was crossed onto *cpk* in part due to the effect of the *wa-2* gene (133), a point mutation in EGFR resulting in decreased tyrosine kinase activity (130), reduces the PKD phenotype in another model for ARPKD, the *orpk* mice. In addition, since *cux-1ΔCR1* mice (37) have a similar phenotype to that of the *wa-2* mice (133) it is possible that Cux-1 is involved in the EGFR pathway. To initially test this idea we examined Cux-1 expression in the presence or absence of EGF stimulation with human epidermal carcinoma cells. Our results here are the first to link Cux-1 within the EGFR pathway. The observation that Cux-1 expression is attenuated in EGF stimulated cells (**Figure 3-3c**) is supported by reports showing EGF stimulation inhibits cell proliferation in A-431 cells (196, 197). Cux-1 is highly expressed when EGFR is not activated by EGF and A-431 cells are undergoing cell division. Therefore, altered Cux-1 expression occurring during the activation of EGFR is consistent with studies demonstrating the expression of Cux-1 in proliferating cells (19, 20, 135). The results here suggest that EGFR activity regulates Cux-1 protein expression.

cux-1ΔCR1 mice carry an in-frame deletion of Cux-1, encompassing exons 10 and 11 (37). These mice display wavy hair and curly vibrissae, and females are unable to support pups because of lactation defects (reviewed in Chapter 1). However, the kidneys of these mice are phenotypically normal. The *cux-1ΔCR1*

phenotype is similar to previously described mouse mutants in the EGFR pathway, including *waved-1* (*wa-1*)(199) and *waved-2* (*wa-2*) (133,200). When examined, however, no change in the expression of EGFR in the *Cux-1ΔCR1* mice was found (37) which is supported by our observations (**Figure 3-3A**). Nevertheless, it is unclear whether Cux-1 is regulated by EGF *in vivo*, or if Cux-1 may target ligands of EGFR. The role of EGFR in polycystic kidney disease has been well described (107, 130, 131, 201,202). Moreover, blocking EGFR activity, either genetically using the *wa-2* mutation (131), or pharmacologically, results in decreased cyst formation and improved kidney function in the *orpk* model for autosomal recessive PKD (203, 204).

To determine whether *cux-1ΔCR1* would similarly alter cyst progression, we crossed the *cux-1ΔCR1/cpk* mice with *cpk* mice to generate *cux-1ΔCR1/cpk* mice. Our results show that an in-frame deletion of Cux-1, encompassing exons 10 and 11 (37), modifies the progression of polycystic kidney disease in *cpk* mice. However, rather than slowing the progression of cystogenesis, the presence of the *cux-1ΔCR1* mutation accelerated cyst growth on a mixed B6/129 background (**Figure 3-5**). Interestingly, the presence of the *cux-1ΔCR1* mutation seems to have no effect on kidney development, as kidneys isolated from *cux-1ΔCR1* mice were phenotypically normal. The absence of a renal phenotype in the *cux-1ΔCR1* mice suggests that the *cux-1ΔCR1/cpk* mutation modifies PKD progression in *cpk* mice. Recently, a candidate modifier gene for the *cpk* mutation was identified. Kinesin family member

12 was mapped to a major effect quantitative trait loci (QTL) complex on chromosome 4 (128). However, Cux-1 is located on chromosome 5 and does not map to a QTL for the modification of renal disease in *cpk* mice (205). Thus, it is unclear to what degree the *cux-1ΔCR1* mutation is a candidate modifier of the *cpk* mutation. It is possible that the *cux-1ΔCR1* mutation perturbs or enhances genes on chromosome 5. Given the nature of the *cux-1ΔCR1/cpk* mice being on a 75% B6/ 25% 129J genetic background it is possible that *cpk* and *cux-1ΔCR1* genes are involved in interactions with any number of additional genes resulting in multifactorial epistasis events.

PKD kidney size and reduced renal function directly relate to the severity of disease (103, 104, 120, 121). The presence of *cux-1ΔCR1* gene with *cpk* results in mice with reduced renal function and larger cystic kidneys. Our results suggest that the *cux-1ΔCR1* gene accelerates cyst growth in *cpk* kidneys, but does not induce cyst formation. There are an equivalent number of cysts in *cpk* and *cux-1ΔCR1/cpk* kidneys. However the existing cysts in *cux-1ΔCR1/cpk* kidneys are in a more advanced stage of development compared to *cpk* cysts. Studies to determine potential mechanism(s) involved in *cux-1ΔCR1* acceleration of cystogenesis and modification of the PKD phenotype are addressed in the ensuing Chapter 4.

CHAPTER FOUR

CUX-1 MODIFICATION OF POLYCYSTIC KIDNEY DISEASE INVOLVES DOWNREGULATION OF THE $p27^{Cip/kip}$ CYCLIN KINASE INHIBITOR

Abstract

Polycystic kidney diseases (PKD) are inherited as autosomal dominant or autosomal recessive traits and are characterized by progressive enlargement of renal cysts. Aberrant cell proliferation is a key feature in the progression of PKD. Cux-1 is a homeobox gene that is related to Drosophila cut, and is the murine homologue of human CDP (CCAAT Displacement Protein). Cux-1 represses the cyclin kinase inhibitors p21 and p27, and transgenic mice ectopically expressing Cux-1 develop renal hyperplasia. However, Cux-1 transgenic mice do not develop PKD. Cux-1 expression is upregulated in cystic kidneys of embryonic *Pkd1* null mice where expression corresponds to cell proliferation. In contrast, Cux-1 expression is not elevated until later stages of cystogenesis in *cpk* mice. Previously we have demonstrated that a 246 amino acid deletion of Cux-1 (termed Cux-1ΔCR1) accelerates PKD in the cpk model for autosomal recessive PKD. Here, we show evidence that downregulation of the cyclin kinase inhibitor p27 occurs in *cux-1ΔCR1/cpk* kidneys. Furthermore, in contrast to cpk kidneys, Cux-1ΔCR1 protein is ectopically expressed in proliferating cyst-lining epithelial cells. This might be possible because Cux-1ΔCR1 protein lacks a Cathepsin-L processing site. Analysis of nuclear Cathepsin-L protein expression levels were inversely correlated with Cux-1 expression. These results suggest a mechanism in which reduced Cux-1 processing

by Cathepsin-L results in the accumulation of Cux-1, down regulation of p27, and increased cell proliferation in ADPKD.

Introduction

The murine transcription factor Cux-1 is structurally related to Drosophila Cut and contains 4 putative DNA binding domains (3 cut repeats, 1 homeodomain) (13, 17, 19, 29). Cux-1 represses the expression of the cyclin kinase inhibitor (CKI) p21 in S phase and is part of the network controlling G1-S transition (55). Cux-1 also represses the CKI p27 (20). Ectopic expression of Cux-1 in transgenic mice results in the downregulation of p27 associated with multiorgan hyperplasia (20). In the kidney, Cux-1 expression is spatially and temporally regulated, with highest expression in the nephrogenic zone, where it is associated with cell proliferation (19, 20). During normal kidney development, p27 is absent from the nephrogenic zone, but is expressed in maturing glomeruli and tubules following the down regulation of Cux-1 (19, 206). Thus, Cux-1 is a cell cycle dependent transcription factor that promotes cell proliferation during the early stages of nephrogenesis by repressing p27 gene expression.

There are several truncated isoforms of Cux-1 (reviewed in Chapter One) in addition to the full-length isoform, called Cux-1 (p200). A nuclear isoform of the cysteine protease cathepsin-L has been identified that proteolytically processes Cux-1(p200) in S-phase (32). While the full-length Cux-1 protein (p200) contains 3 cut repeats and the homeodomain, the proteolytically processed Cux-1 (p110 or p90)

contains only cut repeats 2 and 3, together with the homeodomain (51). Cux-1 (p200) exhibits transient DNA binding activity and functions as a transcriptional repressor, whereas p110 stably binds DNA and can function as a transcriptional activator (51). Cux-1 isoforms develop from alternate splicing (CASP) (31), alternate promoter use (p75) (84), or proteolytic processing (p150) (207). Another Cux-1 transcript, found exclusively in testis, encodes a 55 kD protein, however, it is not known whether it originates by alternate splicing or an alternate promoter (30).

Previously we found that Cux-1 (p200) is highly expressed in cystic kidneys isolated from both *Pkd1* null and *cpk* mice, murine models of ADPKD and ARPKD, respectively (135). In cystic kidneys from *Pkd1* null mice, Cux-1 is highly expressed in both cyst-lining cells and in normal appearing tubule epithelium where expression of Cux-1 is associated with increased cell proliferation. In cystic kidneys from *cpk* mice, Cux-1 is not abnormally expressed until the later stages of cystogenesis (post-natal day 20) where expression of Cux-1 is associated with increased apoptosis in cyst-lining cells. However, CMV/Cux-1 transgenic mice do not develop cystic kidney disease (20), raising the possibility that the upregulation of Cux-1 may be necessary, but not sufficient, for the development of cystic disease.

Tufarelli et al. (37) demonstrated that a mutant form of Cux-1 (termed Cux-1ΔCR1) protein has a higher affinity for biding to DNA compared to wild-type Cux-1. They also demonstrated that Cux-1ΔCR1 protein is expressed at higher levels than wild-type Cux-1 protein within Spleen and Thymus. In Chapter 3, the data demonstrated that *cux-1ΔCR1* accelerated PKD in the *cpk* mouse model. Cysts from

cux-1ΔCR1/cpk kidneys were large, in a more advanced developmental stage compared to cpk cysts, and thus contributed to the larger *cux-1ΔCR1/cpk* size. In this Chapter I will show data engendering correlations with theories which may help to explain the occurrence of accelerated cystogenesis in *cux-1ΔCR1/cpk* mice.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was performed as previously described (44). Kidney sections were immersion fixed in 4% paraformaldehyde and blocked in paraffin. Sections were washed in PBST and blocked in 10% normal goat serum (NGS) at room temperature for 1hour. Rabbit Cux-1 (Santa Cruz), mouse PCNA (Sigma) or Rabbit Cathepsin-L (CALBIOCHEM) primary antibody was applied to sections incubated at room temperature for 1hr at a concentration of 1:100, 1:3000 and 1:50 respectively. Biotinylated goat anti-rabbit (1:400) was used to detect Cux-1 and Cathepsin-L antibody. A horse anti-mouse texas red conjugated secondary antibody (Vector) was utilized to detect PCNA antibody (1:400). Sections were then washed in PBST and incubated with either FITC conjugated avidin (Vector) or incubated with avidin-biotin-peroxidase complex (ABC-Elite: Vector) and DAB. All sections were washed with PBST and then mounted with either Vectashield medium with Dapi (Vector) or dehydrated with graded ethanols, mounted with Permount (fisher), and covered with glass coverslips. All images were captured with an Optronics Magnafire digital camera.

Western Blot Analysis

Human ADPKD and normal human kidney (NHK) were harvested and nuclear extracts prepared as previously described[2]. Briefly, nuclear extracts (45ug) were loaded onto 4-15% SDS-PAGE gels and transferred to PVDF membranes where they were blocked in 5% milk $PBST_{20}$. Membranes were probed with either Cux-1 (1:50), Cathepsin-L (1:2000) or p27 (1:100) (Santa Cruz) primary antibody followed by $PBST_{20}$ washes and HRP-peroxidase (1:10,000) secondary antibody application. Densitometry was performed using Scion Image®. Relative protein level intensity from extracts of three different Cux-1ΔCR1/cpk and cpk animals were normalized for p27 protein analysis.

Apoptosis analysis

Sections were processed for Terminal deoxynucleotidal transferase (TdT)-mediated dUTP-nick-end labeling (TUNEL) with the ApopTag Red InSitu Apoptosis Detection kit (Intergen Co.) according to manufacturer's instructions. Sections were counterstained with DAPI, cover-slipped, and visualized on a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera. Midsagital sections were analyzed for the numbers of positive TUNEL labeled cyst-lining epithelial cells taken as a percentage of all cyst-lining epithelial cells counted within 20 random 40X fields. A Caspase-3 cleaved active form (Calbiochem) detection by western blot was performed using the standard western blot protocol previously described.

Electrophoretic mobility shift assay

Electromobility shift assay was conducted with nuclear extracts from *cpk* and *cux-1ΔCR1* kidneys for binding to p27 promoter and Cux-1 consensus oligonucleotides. Sense and antisense oligonucleotides for p27 (5'CAGCCTGGGGCGGCTCCCGCCGCCGCAACCAAT 3') and Cux-1 consensus DNA binding site (5'AAAAGAAGCTTATCGAT3') were annealed by incubating overnight in a thermocycler (85^0C for 1 sec, 85^0C for 5 min, 20^0C for 6 hrs). The resulting double stranded oligonucleotides included 5' overhanging extensions to facilitate labeling with Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP . The double-strand oligo probes were labeled using the random primed DNA labeling kit (Roche). Unincorporated nucleotides were removed by spin column G25 (Amersham). The counts were measured in liquid scintillation counter ($3X10^4$ cpm were used per 30 μl reaction). Reaction: 5 μg of nuclear extract was added to binding buffer containing 10μg BSA, 1 μg poly(dI–dC) · (dI–dC), 50mM Tris HCl pH 7.5, 250mM NaCl, 25% Glycerol, 5mM DTT, 5mM EDTA in a 30 μl reaction. Then, radiolabeled probe was added and incubated for 30 min at room temperature. For cold competition reaction, 50 molar excess of annealed oligo was added 15 minutes prior to adding the radiolabeled probe. The reaction products were analyzed on a 4% non-denaturing polyacrylamide gel using 0.5x TBE (25 mM Tris-HCl, 25 mM boric acid, 0.5 mM EDTA) as running buffer. Gel was dried and radiolabeled bands were visualized by autoradiography.

Statistics

In all studies, a one-way ANOVA was performed. If significance between the genotypes existed ($P \leq 0.05$) post-hoc analysis by least significant difference (LSD) was performed to determine statistical significance ($p \leq 0.05$) between groups. All statistical analyses were performed using the Statview® statistical program.

Results

Cell proliferation is a key feature of cyst growth (146, 148, 189,208). Moreover, there is a direct correlation between levels of cell proliferation and progression of cystic disease (120, 121, 146, 209,). Thus, one possibility is that the increased cyst size observed in the kidneys from *cux-1ΔCR1/cpk* mice resulted from increased proliferation of the cyst lining epithelial cells. In the developing kidney, Cux-1 is expressed in the nephrogenic zone where it co-localizes with markers for cell proliferation, but is downregulated in maturing glomeruli and tubules (19). However, in *Pkd1* null kidneys, Cux-1 is ectopically expressed in the cyst lining cells, and in normal appearing mature tubule epithelia, where it is associated with cell proliferation (133). **Figure 4-1 d-f** shows that Cux-1ΔCR1 protein co-localized with PCNA in the cyst lining cells of kidneys isolated from 10-day-old *cux-1ΔCR1/cpk* mice. In contrast, wild type Cux-1 protein was not expressed in the cyst lining cells of kidneys isolated from 10-day-old *cpk* mice (**Figure 4-3a-c**), as described previously (133).

The expanded expression of the Cux-1ΔCR1 protein in the cystic kidneys was surprising since this deletion would not be expected to affect the regulatory elements in the Cux-1 gene. Moreover, the levels of Cux-1ΔCR1 mRNA are not different from that of wild type Cux-1 (p200) mRNA (37). However, the levels of Cux-1ΔCR1 protein appear to be elevated in some tissues compared to Cux-1 (p200). To determine whether the Cux-1ΔCR1 protein was elevated in the kidney, we performed Western blot analysis on nuclear extracts from newborn kidneys isolated from wild type and *cux-1ΔCR1* mice. **Figure 4-2A** shows that significantly more of the Cux-1ΔCR1 protein was present in newborn kidneys from *cux-1ΔCR1* mice than the Cux-1 (p200) protein in wild type mice. Furthermore, Cux-1 (p200) was abundantly expressed in human ADPKD cells compared to normal human kidney (NHK) cells (**Figure 4-2B**). There were lower levels of Cux-1 degradation product in ADPKD cells where Cux-1 (p200) was expressed compared to NHK cells. This may suggest that Cux-1 (p200) might be more stable in ADPKD cells in contrast with Cux-1 (p200) in NHK cells.

It is unclear how Cux-1ΔCR1 protein levels are elevated in *cpk* cyst lining cells, within developing kidney, spleen and thymus. Nuclear Cathepsin-L proteolytically cleaves Cux-1 (p200) during S-phase of the cell cycle (32). After

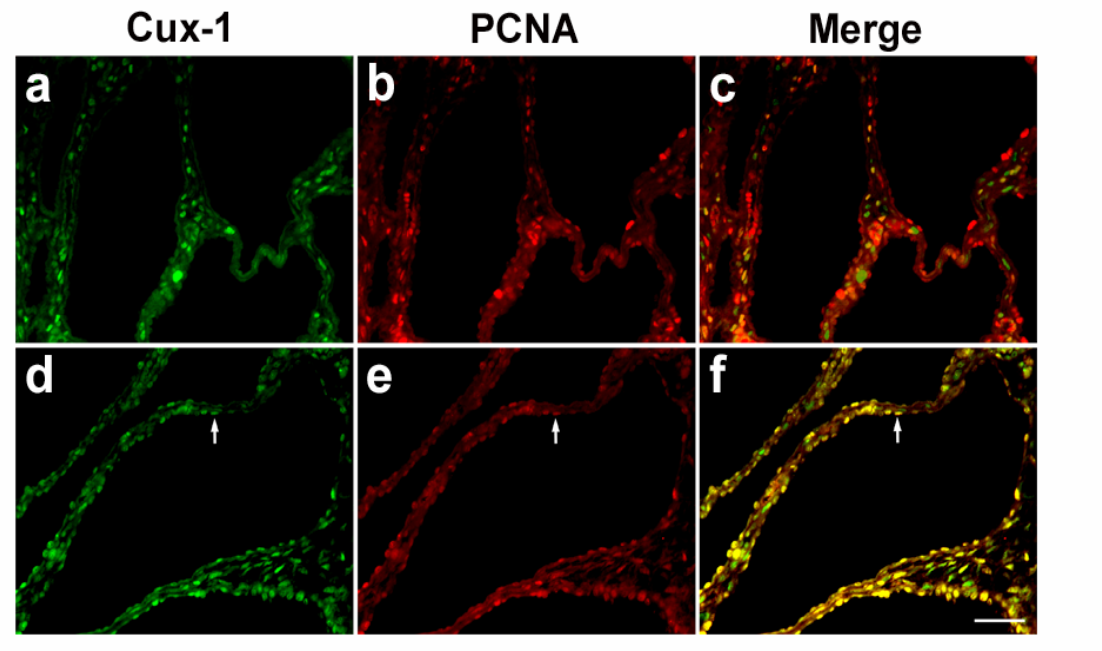


Figure 4-1. Increased proliferation in cystic kidneys from *cux-1ΔCR1/cpk* mice. a-c: Detection of Cux-1(p200) (a) and PCNA (b) in kidneys from 10-day-old *cpk* mice. At early stages of cyst development in *cpk* mice, Cux-1(p200) is expressed in the ***i***nterstitial cells (a), but is absent from the cyst lining cells, where PCNA labeling indicates cell proliferation (b). The merged image (c) shows no overlap of Cux-1(p200) with PCNA. d-f: The mutant Cux-1ΔCR1 protein is expressed in both the interstitial cells and the cyst lining cells (d). The cyst lining cells are proliferative, as indicated by PCNA labeling (e), and the merged image shows co-localization of Cux-1ΔCR1 and PCNA in cyst lining cells (f). Arrows in (d-f) demonstrate co-expression of Cux-1ΔCR1 protein with PCNA in cystic epithelial cells. (a-f) Bar=50 μm.

close evaluation of Cux-1 (p200) sequence compared to Cux-1ΔCR1 we found that the deletion producing the Cux-1ΔCR1 protein contained a Cathepsin-L cleavage site (**Figure 4-3**). Therefore, since Cux-1ΔCR1 protein lacks a Cathepsin-L cleavage site, it might be possible that Cux-1ΔCR1 has a longer degradation half life resulting in the accumulation of Cux-1ΔCR1 protein in PKD. Nuclear Cathepsin-L expression may correlate with Cux-1 (p200) expression. To begin to address this issue, we looked at nuclear Cathepsin-L expression in *pkd1* null kidneys, human ADPKD cystic cells, and cpk kidneys (**Figure 4-4b**). The results indicated that nuclear Cathepsin-L levels are attenuated in PKD1 knockout kidneys and human ADPKD. This is a very interesting finding because it establishes an inverse relationship between Cux-1 (p200) expression levels and Cathepsin-L in wild-type kidney and NHK cells in relation to PKD1 knockout kidney and ADPKD cells (**Figure 4-2** and **Figure 4-4c**). Diminished nuclear Cathepsin-L expression levels in PKD appear to manifest in the ectopic expression of Cux-1 (p200). However, *cpk* kidneys presented with a mild increase in nuclear Cathepsin-L protein expression (**Figure 4-4d**). The mild increase in Cathepsin-L may help provide insight into why Cux-1p200 is not ectopically expressed in *cpk* kidneys. The fact that Cux-1ΔCR1 is ectopically expressed in *cpk* kidneys might be a result of the absence of a Cathepsin-L proteolytic cleavage site in Cux-1ΔCR1 protein.

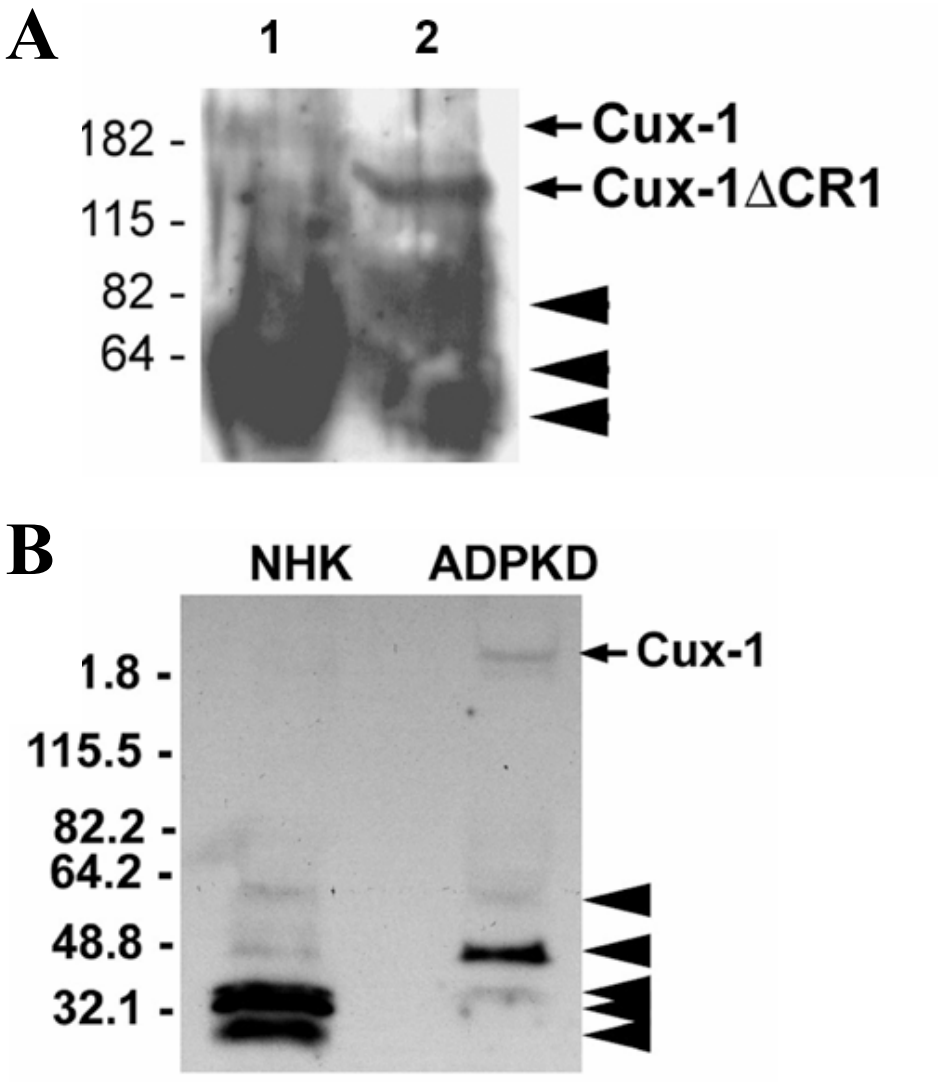


Figure 4-2. Increased Cux-1ΔCR1 protein in developing kidney and downregulation of p27 in *Cux-1ΔCR1/cpk* kidneys. The level of Cux-1(p200) and Cux-1ΔCR1 protein during kidney development were evaluated. (A) Western blot for Cux-1 in kidney nuclear extracts from postnatal-day 7 wild type (lane 1) and *Cux-1ΔCR1* (lane 2) mice. There is significantly more Cux-1ΔCR1 in developing kidneys from *Cux-1ΔCR1* mice than Cux-1(p200) in developing kidneys from wild type mice. (B)The levels of Cux-1(p200) were evaluated in normal human kidney (NHK) and human ADPKD cells. Cux-1(p200) is abundantly expressed in ADPKD. (◄) Denotes Cux-1 degradation products.

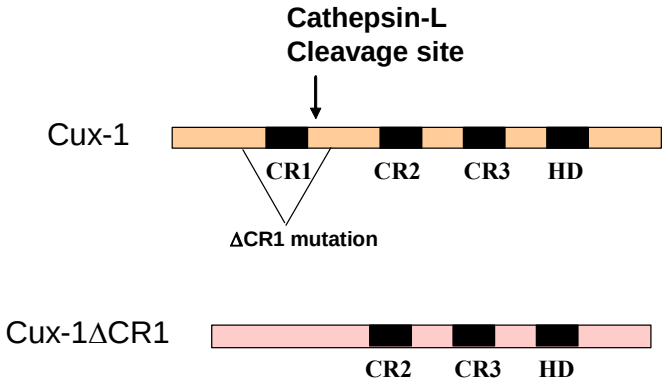


Figure 4-3. Cux-1ΔCR1 protein lacks a Cathepsin-L cleavage site.

Cux-1 (p200) protein when overexpressed in transgenic mice results in renal hyperplasia associated with the downregulation of p27 cyclin kinase inhibitor. The p27 gene is a target for Cux-1(p200) mediated repression. Therefore, to determine whether the increased expression of the Cux-1ΔCR1 protein in *cpk* mice altered the expression of p27, we compared the expression of p27 in kidneys isolated from *cux-1ΔCR1/cpk* and *cpk* mice (**Figure 4-5A**). We found that p27 expression was reduced by two-fold in the kidneys from *cux-1ΔCR1/cpk* mice in comparison to *cpk*. Since Tufarelli et al (37) demonstrated that Cux-1ΔCR1 protein binds to promoter DNA with a higher affinity then Cux-1(p200) we wanted to evaluate the ability of Cux-1ΔCR1 to bind p27 promoter in *cpk* kidneys. We found that there was more Cux1ΔCR1protein bound to the p27 promoter in comparison to Cux-1(p200) within cpk kidneys (**Figure 4-5B**).

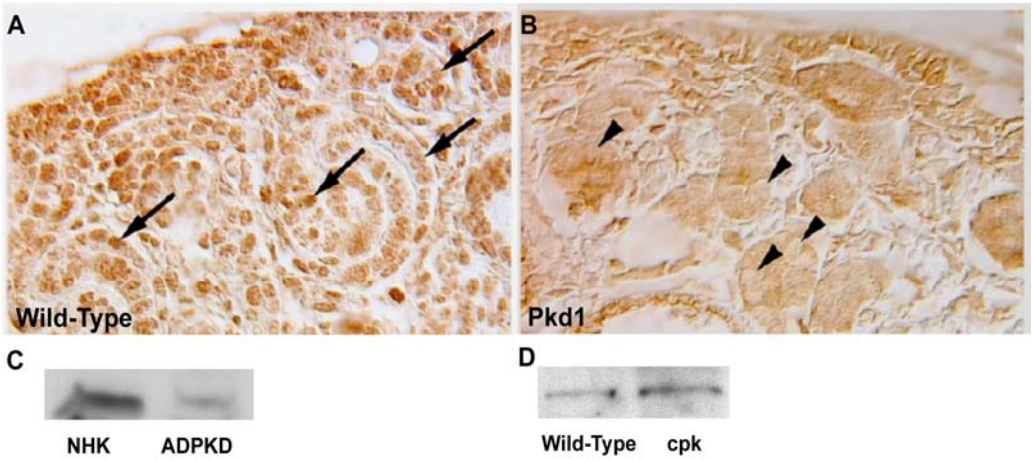


Figure 4-4. Abnormal nuclear Cathepsin-L expression in PKD. (A) wild-type embryonic kidneys express Cathepsin-L within the nucleus of cells (🡐). (B) Age matched 18 day embryonic kidney from PKD1 knockout mice. Cathepsin-L expression is less abundantly nuclear and can now be observed in the cytoplasm (◀). (C) Western blot for Cathepsin-L within nuclear extracts of NHK and ADPKD show reduced nuclear Cathepsin-L in ADPKD cystic cells. (D) Western blot for Cathepsin-L within nuclear extracts from wild-type and *cpk* kidneys at post-natal day 3 revealed modest elevation of nuclear Cathepsin-L levels in *cpk* kidneys.

While there is limited apoptosis in the cysts of 10-day-old *cpk* mice, apoptosis is increased at later stages of cyst growth, where it is thought to contribute to cyst progression (135, 140, 209). Moreover, inhibition of apoptosis slows cyst progression in mouse and rat models of PKD (140,209). Consistent with an acceleration of cyst progression in the kidneys of *cux-1ΔCR1/cpk* mice, we observed an increase in TUNEL labeling in the cystlining cells of *cux-1ΔCR1/cpk* mice

compared to *cpk* mice, and an increase in activated Caspase-3 in kidneys from *cux-1ΔCR1/cpk* mice compared to *cpk* mice (**Figure 4-6A-D**).

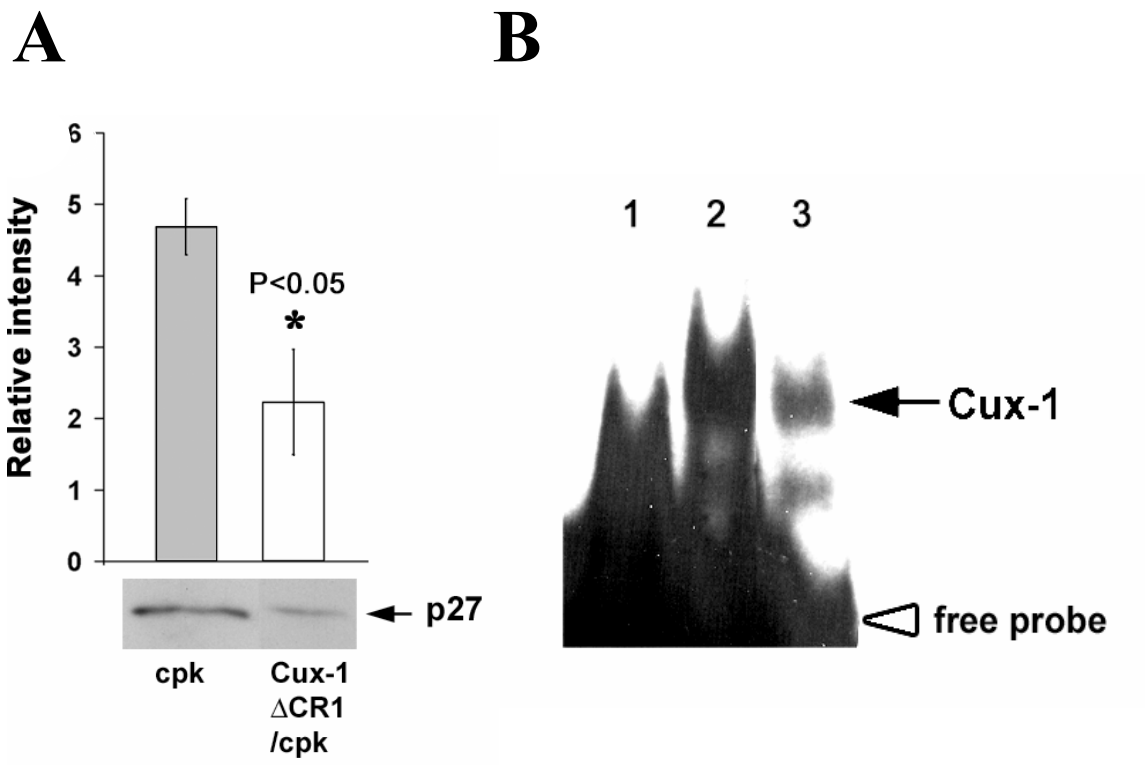


Figure 4-5. Cux-1ΔCR1 protein binds to the p27 promoter when p27 is downregulated in *cpk* kidneys. (A) Western blot for p27 in kidneys from 10-day-old *cpk* and *cux-1ΔCR1/cpk* mice, and mean relative intensity ± SEM of p27 levels. (*) Denotes a significant reduction in p27 protein levels in the kidneys of *cux-1ΔCR1/cpk* mice compared to *cpk* mice. (B) EMSA performed on cpk (A p27 promoter sequence (lanes 1 and 2) and an oligonucleotide control sequence, known to bind Cux-1 (lane 3) were incubated with *cpk* (anes 1 and 3) or *cux-1ΔCR1/cpk* (lane 2) nuclear extracts. There were more abundant levels of Cux-1ΔCR1 protein bound to p27 promoter (lane 2) compared to wild-type (lane 1).

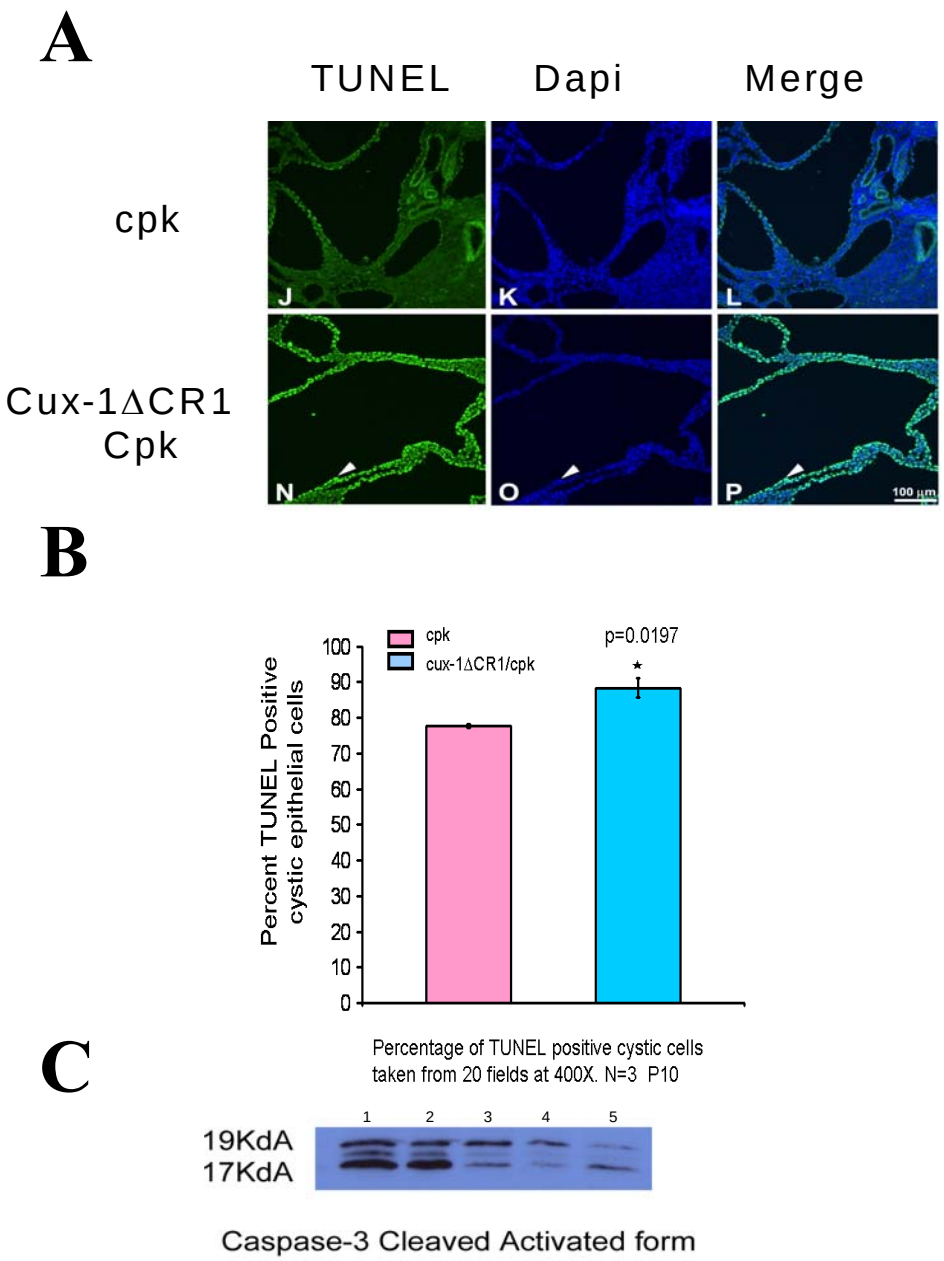


Figure 4-6. Increased apoptosis occurs in *cux-1ΔCR1/cpk* mice. (A) TUNEL labeling is increased in cyst lining cells of *cux-1ΔCR1/cpk* mice (p) compared to *cpk* kidneys (L). (B) Percent TUNEL positive cystic cells are elevated in ***cux-1ΔCR1/cpk*** kidneys**.** (C) Levels of activated caspase-3 are increased in cystic kidneys from *cux-1ΔCR1/cpk* mice (lanes 1-2) compared to *cpk* mice (lanes 3-5).Bar, 100 µm.

Discussion

We previously showed that Cux-1 is ectopically expressed in cystic kidneys isolated from *Pkd1* null and *cpk* mice (135). In *Pkd1* null mice, Cux-1 was ectopically expressed in cyst lining cells and in what appeared to be normal differentiated tubules, and was associated with abnormal cell proliferation. In contrast, Cux-1 was not ectopically expressed in cystic kidneys isolated from 7- or 9-day-old *cpk* mice (135). Rather, Cux-1 was upregulated in cystic kidneys from 20-day-old *cpk* mice (19, 135), when cystogenesis was highly advanced, and there was little parenchyma remaining. Moreover, the expression of Cux-1 in the cysts was more closely associated with apoptosis than with cell proliferation. The difference in Cux-1 expression between these two animal models of PKD is unclear, but suggests that the molecular mechanisms of cyst growth between the *Pkd1* null and *cpk* mice are different.

Cystic epithelial cells are thought to undergoe dedifferentiation and aberrant cell proliferation. Bhunia et al. (148) demonstrated that the Polycystin-1 protein, where mutation is involved in ~85% of human ADPKD cases, regulates the cyclin kinase inhibitor p21. The ectopic expression of Cux-1ΔCR1 protein in cyst lining epithelial cells may play a role in cell proliferation leading to increased numbers of cysts in a more advanced developmental stage (**Figure 4-1**). Tufarelli el al.(37) found that Cux-1ΔCR1 protein is upregulated in Spleen and Thymus. It is unclear how Cux-1ΔCR1 protein levels are elevated compared to wild-type Cux-1 (p200) protein.

Cathepsin-L is involved in initiating the Cux-1 degradation pathway (32, 51). Our results revealed that in human ADPKD cystic cells and PKD1 knock out mice, increased levels of full-lenth Cux-1 (p200) protein were associated with reduced levels of nuclear Cathepsin-L (**Figure 4-2** and **Figure 4-4**). It is possible that increased amounts of Cux-1 (p200) lead to reduced nuclear Cathepsin-L levels which are then unable to initiate the proteolytic degradation pathway. Nuclear Cathepsin-L levels are relatively elevated in *cpk* kidneys (**Figure 4-4C**) when Cux-1 (p200) expression is restricted to interstitial cells which may help to explain how Cux-1 (p200) protein is not expressed in cystic epithelial cells. However, Cux-1ΔCR1 protein is ectopically expressed in cyst lining epithelial cells (**Figure 4-1**). One possibility is that since Cux-1ΔCR1 protein lacks a Cathepsin-L cleavage site (**Figure 4-3**), Cux-1ΔCR1 protein might avoid Cathepsin-L mediated proteolytic cleavage. This may result in increased amounts of Cux-1ΔCR1 protein levels in *cpk* kidneys.

Cux-1 overexpression in transgenic mice results in renal hyperplasia and downregulation of p27 (20). We previously showed that p27 is upregulated in kidneys isolated from *cpk* mice, compared to age matched wild type mice (44). Therefore the observation that p27 protein expression levels were attenuated in *cux-1ΔCR1/cpk* kidneys (**Figure 4-5**) may provide insight to why these mice have such a severe *cpk* phenotype in regard to aberrant cell proliferation. We have previously shown that Cux-1 (p200) represses p27 promoter activity in a concentration dependent manner (20). The decreased expression of p27 in cystic kidneys

expressing the Cux-1ΔCR1 protein suggests that this protein can function similar to the Cux-1 (p200) protein. Since Cux-1ΔCR1 protein in *cpk* kidneys binds more abundantly to p27 promoter (**Figure 4-5b)**, which correlates with p27 downregulaton (**Figure 4-5a**), it is possible that increased Cux-1ΔCR1 protein facilitates p27 down regulation and rapid progression through the cell cycle resulting in increased cell proliferation and severe PKD observed within Chapter 4.

Cystic epithelial cells undergoe apoptosis in *cpk* mice (140, 209). Cux-1 is not abnormally expressed until the later stages of cystogenesis (post-natal day 20) where expression of Cux-1 is associated with increased apoptosis in cyst-lining cells (135). Moreover, Ali et al. (136) showed that Caspase-3 activity is active during post-natal day 20 confirming that increased Apoptosis is occurring during the more advanced stages of the disease. Furthermore, Tao et al. (209) demonstrated that *caspase-3* gene knock out when crossed with *cpk* slows *cpk* disease progression. Therefore, increased Caspase-3 activity and apoptosis in *cpk* kidneys correlate with increased *cpk* cystic disease progression. Reduced TUNEL labeling and increased Caspase-3 expression in *cux-1ΔCR1/cpk* kidneys further supports data that *cux-1ΔCR1/cpk* mice have severe PKD (Chapter 3). The mechanism behind apoptosis in the *cpk* model and PKD in general is unclear. In contrast to the results from Tao et al. (209) which demonstrated that gene reduction of Caspase-3 ameliorates PKD, Ostrom et al. (137) reported that reduced *Pax2* gene dosage increases apoptosis and slows the progression of renal cystic disease. The balance between apoptosis and cell proliferation may contribute to the net effect of *cux-1ΔCR1/cpk* kidneys being

larger than *cpk* kidneys. Enhanced apoptosis during advanced stages of PKD might be a biological response to try to limit aberrant cell proliferation. However, the role of apoptosis beyond a marker for advanced cystic disease is poorly understood.

The role of Cathepsin-L in regulating ectopic expression of Cux-1 during PKD requires further investigation and will be a topic for discussion in Chapter 5. The results presented in Chapter 4 show that cyst-lining epithelial cells ectopically express Cux-1ΔCR1 protein and are undergoing cell proliferation, which may contribute to the accelerated PKD cyst maturation in *cux-1ΔCR1/cpk* kidneys observed in Chapter 3. Downregulation of the cyclin kinase inhibitor p27 within *cux-1ΔCR1/cpk* mice may be linked to a possible increase in cell proliferation which may be responsible for advanced PKD. Since Cux-1ΔCR1 protein binds to p27 promoter more abundantly than wild-type Cux-1 (37) and is associated with p27 downregulation in *cpk* kidneys (**Figure 4-5**), a direct link between Cux-1 and p27 in regulating PKD has now been identified.

CHAPTER FIVE

DISCUSSION, SUMMARY AND CONCLUSIONS

The majority of information regarding Cux-1 function and expression pattern has come from studying the homologue of Cux-1 that is present in Drosophila which is called "Cut" (1, 3, 4). The Malpighian tubules serve to provide renal function to the fly. Mutational studies involving the drosophila *cut* gene demonstrated that mutations in *cut* result in defects in malphigian tubules ranging from moderate (defective morphogenesis) to severe (absence of malphigian tubules) (5-8). Cux-1 is highly expressed in induced and uninduced mesenchyme, as well as in ureteric bud epithelium and S-shaped bodies in murine kidneys (19). The involvement of Cux-1 in signaling pathways during kidney development in mammals is poorly understood. During wing development in Drosophila, multiple genetic interactions between Cut, Notch and the wnt/Wingless signaling pathways have been reported (reviewed in 13).

Previously, work from our lab has demonstrated that in the developing kidney, Cux-1 expression overlaps with that of Notch receptors (Notch 1-4) and ligands (Jagged-1,-2, and Delta 1) in the condensing mesenchyme, in pretubular aggregates (comma and S-shaped bodies), and in developing podocytes of capillary loop stage glomeruli (70) . Furthermore, Cux-1 was significantly up-regulated in a rat kidney epithelial cell lines (RKE) which constitutively express the Notch 1 intracellular domain conferring active Notch 1 signaling. RKE cells have elevated Cux-1 protein levels present with reduced levels of p27 protein. Moreover, Cux-1

interacts with the Groucho homolog TLE-4, a corepressor recruited by Notch effector proteins. These results suggest that Cux-1 may function in the context of the Notch signaling pathway during embryogenesis within developing kidneys. However, evidence of Notch signaling through Cux-1 has not been directly demonstrated as of this report.

Cux-1 contains Ser and Thr residues within its cut repeat (CR) domains (CR1, CR2, CR3) as well as within its homeodomain (HD) which are targets for phosphorylation and dephosphorylation events as was described in Chapter 1. PKC phosphorylation sites include Thr 415 (CR1 domain), Thr 804 (CR2 domain) and Ser 987 (CR3 domain) (60). Casein kinase II phosphorylates Ser 400 (CR1 domain), Ser 789 (CR2 domain) Ser 972 (CR3 domain) (59). Cyclin A-CDK1 phosphorylates Ser 1237 (inbetween HD and CR3 domains) and Ser 1270 (within HD domain) (62). PKA phosphorylates Ser 1215 (located between the CR3 and HD domains) (61). Coqueret et al. demonstrated that cdc25 phosphatase activity dephosphorylates Cux-1 which permits Cux-1 DNA binding and implicated Cux-1 as cell-cycle dependent transcription factors.

Phosphorylation controls the activity of transcription factors and co-regulators at different levels via the following: 1) adjusting the activity of chromatin remodeling complexes or enzymes that modify the chromatin package, 2) influencing the stability of transcription factors and their regulators, 3) restraining the subcellular localization of transcription factors, 4) Modulating protein-protein interactions among transcription factors , coregulators and components of the basal

transcription complexes, and 5) altering the ability of transcription factors to bind to DNA (Reviewed in 210-214). Numerous examples have been documented regarding the phenomenon of post-translational modification of transcription factors (Reviewed in 210-214). Rapid post-translational modifications are very important during the cell-cycle and are known to regulate the effects of many transcription factors. For example, phosphorylation of the retinoblastoma (RB) protein results in a change in conformation removing RB ability from stably binding to E2F protein and thus permitting E2F to be released and function as a transcription factor (213). Gupta et al. (76) found that RB is a co-repressor protein that interacts with Cux-1 to regulate cell-cycle controlled histone H4 transcription. They found that Cux-1 functions as the DNA binding subunit of the cell cycle controlled HiNF-D complex which is involved in regulating cell proliferation. HiNF-D interacts with all five classes (H1, H2A, H2B, H3, and H4) of the cell-cycle dependent histone genes. These histone genes are important in gene regulation and are transcriptionally and become activated at the G(1)/S phase transition independent of E2F activation. RB is an intrinsic component of the HiNF-D complex. Gupta et al. (76) found that the Cux-1/RB interaction, similar to the E2F/RB interaction, occurs within the A/B large pocket (LP) of pRB. Therefore it is conceivable, similar to how RB phosphorylation leads to conformational changes in RB resulting in the release of E2F factor, that phosphorylation/dephosphorylation of RB and/or Cux-1 may also affect RB/Cux-1 protein-protein interactions.

We previously demonstrated that Cux-1 can bind to TLE-4 co repressor protein which is involved in transcriptional protein complex downstream of the Notch pathway (70). Many transcription factors, (such as AP-1, HiFs and homeodomain box containing proteins) exist as protein homodimers or heterodimers or form complexes with many additional proteins (211, 212 for review). The population of transcription factor binding partners at a given time or place during the cell-cycle appears to coordinate cell-cycle progression. Cux-1 may exist in a complex with additional proteins such as TLE-4 in addition to interacting with other completely different proteins such as with RB in the HinF-D complex (76). Therefore it appears that Cux-1 may form different protein-protein complexes possibly in different cell-types during distinct time periods during development. Determination of the *in vivo* Cux-1 phosphorylation state during different time points in development has not yet been investigated. Therefore Cux-1 function in regard to post-translational modification is still not clear within the developing kidney.

CamKII, PKC, Cyclin-A CDK1 and cAMP-mediated PKA activity are all mitogenic factors that phosphorylate Cux-1 (59-62). Since phosphorylation of Cux-1 is associated with reduced Cux-1 DNA binding ability, the observation that these mitogenic factors could possibly prevent Cux-1 binding and repression of target promoters such as the cyclin kinase inhibitors p21 and p27, whose repression favors accelerated cell-cycle progression, is quite puzzling. However, the majority of the studies that identified phosphorylation events occurring within Cux-1 were performed *in vitro* in immortalized cells which may not provide full detail to what is

occurring within living kidney tissue. Furthermore, some of these studies were performed in mammary tissue instead of within kidney tissue (61). Residue specific Anti- phospho Cux-1 antobodies need to be generated to observe Cux-1 phosphorylation state *in vivo* during different stages of kidney development.

Cux-1 phosphorylation/dephosphorylation has been implicated in regulating the ability of Cux-1 to bind to DNA and thus regulate transcription of its gene targets (59-62). Based upon what is known about transcription factors and post-translational modifications of transcription factors (reviewed in 210-214), it is possible that Cux-1 phosphorylation state determines Cux-1 binding partners within the nucleus. For example, Cux-1 in a phosphorylated state may discriminately interact with nuclear proteins which mask Cux-1 DNA binding domains in 3-dimensional conformation. In contrast, when Cux-1 is in a dephosphorylated state it may interact with a different set of co-repressor proteins which do not mask Cux-1 DNA binding domains and in fact may enhance the availability of those domains to be recognized by DNA. An additional possibility is that when Cux-1 is in a phosphorylated state it is targeted for proteolytic cleavage more readily as a result of a conformational change that might unmask cleavage sites within Cux-1. These possibilities are supported by Harada et al. (215) who recently found that Cux-1 p110 Cathepsin-L proteolytic cleavage product is recruited to the promoter of cell cycle-related targets preferentially during S phase of the cell-cycle. Therefore the idea that post-translational modification of Cux-1 may alter recruitment of different transcriptional machinery to the site of a target promoter is strengthened.

The Cux-1 p110 protein lacks the CR1 domain which has been suggested to be an "autoinhibitory domain" involved in repressing the DNA binding ability of CR2 CR3 and HD (52). Yet it is also possible that the CR1 domain is structurally masking the CR2 CR3 and HD domains and thus preventing these domains access to DNA. The C-terminal region of Cux-1 contains two active repression domains that can recruit histone deacytlase activity (48, 56). Another possibility for how regulation of Cux-1 may affect Cux-1 function is that the cleavage of full length Cux-1 p200 to Cux-1 p110 provides greater access to the two C-terminal active repression domains within Cux-1 which may enhance repression. Generation of a transgenic mouse that overexpresses Cux-1 p110 protein might be useful to determine the *in vivo* effect of Cux-1 proteolytic cleavage.

The regulation of Cux-1 function may be further complicated by the possibility that Cux-1 intra- and inter protein conformational changes could be dependent upon site specific residue phosphorylation/dephosphorylation. Residue specific phosphorylation has already been demonstrated both *in vitro* (59, 60, 62) and *in vivo* (61) within Cux-1. PKC, PKA, CamKII, phosphorylate different residues within Cux-1 (59-61). PKC and Casein kinase II target residues within the CR1 domain which are not present in Cux-1 p110 (59, 60). Since single phosphorylation events within a protein are known to modify protein conformation, it is conceivable that the lack of signaling to these resides may also contribute to Cux-1 p110 recruitment to the promoter. Therefore, Cux-1 protein intramolecular and intermolecular protein interactions may also play a role in transcriptional activity.

The extent to which Cux-1 structural alterations occur in regard to proteolytic cleavage and phosphorylation/dephosphorylation are experiments that merit future investigation.

The model that we proposed for Cux-1 function during kidney development (**Figure 1-12**) involves the binding of Cux-1 to p27 promoter to repress transcription in a Cux-1 concentration dependent manner. However, based on what is understood about the process of transcriptional regulation by transcription factors (210-214) the observations of full length Cux-1 phosphorylation and dephosphorylation may only tell us part of the story. Post-translational modification such as phosphorylation, acetylation, and proteolytic cleavage may play a role in mediating Cux-1 DNA binding ability (reviewed in 13), it is not clear how direct post-translational modification alters DNA binding in relation to additional protein-protein interactions occurring *in vivo* during kidney development. Moreover, a comprehensive study on Cux-1 target gene promoter binding interactions and protein-protein interactions, during the various stages of kidney development has yet to be performed.

One of the goals of my dissertation work was to learn about the effect of Cux-1 on kidney development. In Chapter 2, I discovered how inhibition of a serine/threonine phosphatase, Calcineurin, can reduce cell proliferation in developing metanephric kidneys. Calcineurin inhibition by treatment with Cyclosporin A resulted in increased amounts of phosphorylated Cux-1 (**Figure 2-6**) correlated with decreased cell proliferation (**Figure 2-2**) and increased p27 (**Figure 2-8**) expression within the nephrogenic zone where Cux-1 is normally expressed

(**Figure 1-11**). These results suggest that Cux-1 in a phosphorylated state may be unable to repress p27 cyclin kinase inhibitor expression. Calcineurin A α knockout mice had the same phenotype as that of CSA treated metanephric kidneys (**Figure 2-1** versus **Figure 2-2**). However, the phosphorylation state of Cux-1 in Calcineurin A α knockout kidneys was not evaluated.

Potential experiments derived from my studies in Chapter 2 should involve the direct testing of Cux-1 phosphorylation and dephosphorylation in mediating kidney development. The best way to do this would be to develop mice with Alanine point mutations in place of the Ser and Thr residues that undergoe phosphorylation dephosphorylation within Cux-1. Individual Alanine amino acid replacement mice would be necessary to determine the extent of each residue involved in regulating kidney development. Another interesting experiment would be to determine by immunoprecipitation, the amount of phosphorylated Cux-1 present within the Cux-1 CMV transgenic kidneys. The phenotype of Cux-1 CMV transgenic mice (20) may in fact be a consequence of reduced phosphorylated Cux-1 protein. Evaluation of Cux-1 phosphorylation levels versus unphosphorylated levels in transgenic mice need to be performed. Experiments in the distant future might center on developing an easily phosphorylated Cux-1 construct that can be overexpressed in mice and cell lines. It would also be interesting to see if the phenotype is opposite to that of the Cux-1 CMV transgenic mice.

The results from Chapter 2 imply that Calcineurin via a direct or indirect mechanism is involved in the regulation of Cux-1 phosphorylation/dephosphorylation (**Figure 5-1**). This is a very significant finding because it is the first direct evidence to establish a required pathway necessary for normal mouse kidney development involving Cux-1. An *in vitro* experiment involving purified phosphorylated Cux-1 protein in the presence of activated Calcineurin should be performed to determine if Calcineurin can directly dephosphorylate Cux-1.

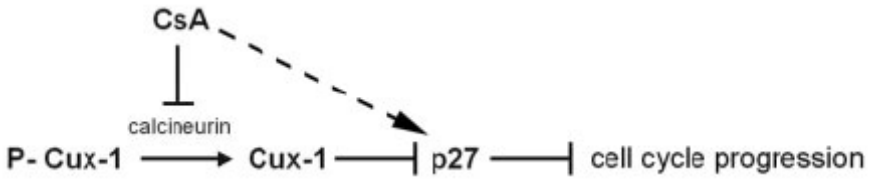


Figure 5-1. A potential model of Cux-1 regulation by calcineurin. Our previous results show that Cux-1 down-regulates the expression of the cyclin kinase inhibitor p27 (20). Because p27 induces cell cycle arrest, increased Cux-1 expression or activity would result in increased cell cycle progression. Our present results show that inhibition of the phosphatase calcineurin by cyclosporin A (CsA) results in increased phospho-Cux-1, which is unable to bind DNA (59, 60), and suggests that Cux-1 activity may be regulated by calcineurin. In addition to down-regulation of p27 by Cux-1, CsA treatment may also result in p27 activation independent of Cux-1. Image taken from Alclay et al. (242).

Calcineurin phosphatase activity requires an increase in intracellular Calcium levels sufficient enough to bind Calmodulin (Reviewed in 170). Calcium-Calmodulin in turn will form a complex with Calcineurin A (phosphatase activity) and B (regulatory) subunits and will then target serine and threonine residues on

target proteins for dephosphorylation. It is not clear which serine and threonine residues of Cux-1 are targeted for dephosphorylation as a result of the Calcineurin pathway during kidney development. Experiments involving the design of mice with point mutation replacement of distinct residues targeted for phosphorylation may provide further insight into the role of phosphorylation/ dephosphorylation during kidney development.

Polycystic kidney disease (PKD) is considered a developmental disorder. The study of primary apical cilia involved in the process of cystogenesis in PKD has been an area of great interest (185, 187). The observation that all proteins that when mutated results in Polycystic Kidney Disease (Autosomal recessive and Autosomal dominant) co-localize to the nephron tubular primary apical cilia has lead to different thoughts on the manifestation of PKD (110). Although ARPKD and ADPKD result from mutations in entirely different genes in rodents and man (98, 101), the phenomenon of cystogenesis may involve primary apical cilia common pathway (110, 185, 187). It is thought that tubular urinary flow is detected by primary apical cilia bending which results in a mechano-signal transduction event involving a conformational change within Polycystin-1 protein which interacts with Polycystin-2 through a C-tail, leading to the opening of the Polycystin-2 channel and the influx of Calcium into the cell (reviewed in 187). The influx of intracellular calcium is then thought to maintain the nephron epithelial cell in a "differentiated state" while mutation in Polycystin proteins lead to dedifferentiation, cell proliferation, and fluid secretion leading to cyst development (111, 216-220). This may involve the

activation of genes by Calcium pathways which require further investigation. Mutation within PKD gene products, which serve as machinery for proper ciliary bending mechanosignal transduction, may lead to decreased intracellular Calcium entry into the cell as a result of a failure of proper Polycystin-1 regulation of Polycystin-2 Calcium channel activity (187, 216). The consequences of decreased intracellular calcium may trigger nephron epithelial cells to be in a dedifferentiated state resulting in an alteration of cell polarity and aberrant cell proliferation (216). Regardless of what possible mechanism(s) are driving cystogenesis, there is sufficient evidence linking alteration of intracellular calcium to cystogenesis and ciliary mechanosignal transduction (216-222).

This idea that PKD is a result of a defect in intracellular calcium levels (reduced calcium) ties in nicely with reports linking Polycystin-1 to the regulation of Polycystin-2 Calcium channel activity (223). Moreover, Puri et al. (221) demonstrated that Polycystin-1 can activate the calcineurin/NFAT (nuclear factor of activated T-cells) signaling pathway through through the heterotrimeric Gα protein, (Gq) and mediate activation of phospholipase C (PLC). They proposed a model whereby Polycystin-1 signaling leads to a sustained elevation of intracellular Ca^{+2} mediated by Polycystin-1 activation of Galpha(q) followed by PLC activation, release of Ca^{+2} from intracellular stores, and activation of store-operated Ca^{+2} entry, thus activating calcineurin and NFAT.

The role of Cux-1 in kidney development is to regulate cell cycle progression through the repression of the cyclin kinase inhibitors p21 and p27 (Chapter 1). Cux-

1 is highly expressed in proliferating cells during kidney development (19, 20). A hallmark of PKD is aberrant cell proliferation. Since Cux-1 is developmentally expressed in proliferating cells and PKD is considered a developmental disorder involving renal tubular dedifferentiation and cell proliferation, the role of Cux-1 in PKD was investigated. My results (Chapter 2) demonstrate that Calcineurin regulates Cux-1 phosphorylation state during kidney development. Activation of Calcineurin activity requires elevated levels of intracellular Ca^{+2} (170). Therefore, since inhibition of Calcineurin activity results in elevated phospho Cux-1 levels (Chapter 2), decreased intracellular calcium could lead to an increase in phosphorylated Cux-1. However, it is not known if phosphorylation/dephosphorylation regulates Cux-1 function in PKD. It is conceivable that the regulation of Cux-1 function during kidney development and in PKD is different in regard to the availability of potential protein substrates and signaling pathways. This idea is supported by the observation that Cux-1 is not expressed in proliferating cells during early PKD in *cpk* cystic kidneys yet is ectopically expressed in *Pkd1* knockout cystic kidneys during early development (135).

Cux-1 is ectopically expressed in *Pkd1* knockout mice (135), *cpk* mice (19) and in human ADPKD (**Figure 4-2**). It is possible that ectopically expressed Cux-1 protein levels serve to enhance mitotic signaling pathways by indirectly binding to transcriptional complexes such as RB/HinF-D, as it may normally function during G1-S-phase transition of the cell cycle (66-69). In the *cpk* mouse, Cux-1 expression

is restricted to interstitial cells and does not co-localize in proliferating cyst lining epithelial cells during the early stages of the disease (135). During the later stages of *cpk* disease (3 weeks), Cux-1 is ectopically expressed and co-localizes with proliferating cells (135). This may indicate that as the disease progresses, Cux-1 may participate in cell proliferation. However, Cux-1 positive cyst-lining epithelial cells undergoe apoptosis at 3 weeks when the cyclin kinase inhibitors p21 and p27 are upregulated (135). Upregulation of p21 and/or p27 may trigger apoptosis in *cpk* kidneys however that possibility has not been investigated. It is also possible that there is decreased Calcineurin activity within *cpk* kidneys which could result in Cux-1 in a phosphorylated state and unable to repress p21 and/or p27 gene expression. Although this is unclear and Calcineurin activity within *cpk* kidneys has not been evaluated. During kidney development, upregulation of Cux-1 is correlated with downregulation of the cyclin kinase inhibitors p27 and is highly expressed in proliferating cells (19, 20). Therefore, Cux-1 appears to function differently during kidney development and within the *cpk* model for PKD.

Since Cux-1 is upregulated in PKD (19) previous work from our laboratory evaluated the overexpression of Cux-1 in transgenic mice which resulted in renal hyperplasia without the development of cystic kidneys (20). This observation suggested that Cux-1 may be necessary in PKD in regard to possibly contributing to cell proliferation but not sufficient by itself for the development of polycystic kidneys. It was this idea coupled with the observation that overexpression of Cux-1 in transgenic kidneys resulted in the downregulation of p27 (20) which became the

rationale for studying the effect of Cux-1 mutation in PKD. Since overexpression of Cux-1 represses p27 expression, we predicted that the Cux-1 to p27 pathway may function as a "modifier" pathway involved in exacerbating or ameliorating PKD depending upon upstream factors that regulate Cux-1 expression and function. Prior to the conclusion of my experiments in Chapter 3 there was no evidence that Cux-1 was directly involved in PKD. When I crossed the *Cux-1ΔCR1* gene onto *cpk* mice I found that *Cux-1ΔCR1* accelerated PKD cystogenesis resulting in severe PKD (**Figure 3-5**). These results established that Cux-1 contributes to PKD. Moreover, since *Cux-1ΔCR1* gene alone does not result in cystic kidneys (37) but when present with the *cpk* gene accelerates disease (Chapter 3), the *cux-1ΔCR1* gene can be considered a potential candidate modifying gene for PKD (**Figure 5-2**).

The future directions from my studies in Chapter 3 would have to center around evaluation of the *Cux-1ΔCR1/cpk* phenotype on a congenic B6 background. The reason why this is necessary is because the mixed B6/129 background by which the animals in this chapter were analyzed on, may underscore the effect of a plethora of unknown genetic interactions that could act additively or subtract from the true effect of the *Cux-1ΔCR1* genetic interaction with *cpk* (See 224 for review of effect of genetic background). The *cpk* gene may be involved in genetic interactions with genes on the 129 background. Therefore, analyzing this phenotype when *Cux-1ΔCR1* is present on B6 with *cpk* may uncover with greater clarity the *Cux-1ΔCR1/cpk* genetic interaction.

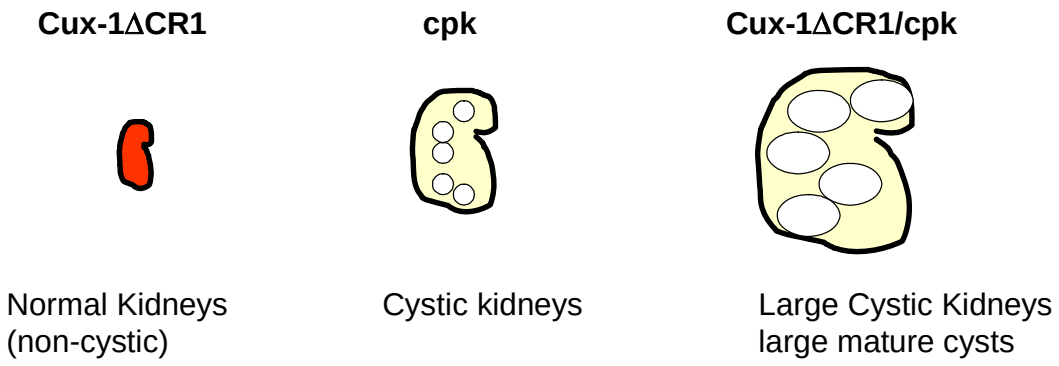


Figure 5-2. *cux-1ΔCR1* is a modifying gene for Polycystic Kidney Disease. Mice carrying the *Cux-1ΔCR1* gene have normal kidneys. In contrast mice carrying the cpk gene develop polycystic kidneys. The presence of both the *Cux-1ΔCR1* and *cpk* genes within mice results in a more severe form of polycystic kidney disease.

Tazón-Vega et al. (225) studied potential candidate modifying genes in ADPKD patients with mutations in the *PKD-1* gene and were not able to identify a modifying gene for the disease. They saw that angiotensin converting enzyme (ACE) had only a slight influence on the age of onset of end stage renal disease for PKD-1 mutant patients. However, Konoshita et al. (226) found that ACE polymorphism led to a more rapid onset of end stage renal disease. Tazón-Vega et al. (225) were not able to correlate polymorphisms of epidermal growth factor receptor and TGFβ as being modifier genes. Magistroni et al. (227) found that EGFR polymorphisms lead to earlier end stage renal disease but their data was not statistically significantly. The cystic fibrosis (CF) gene which encodes the cystic fibrosis transmembrane conductance regulator (CFTR) was also studied in human patients and the presence of CF mutations within ADPKD patients were not statistically significant (228). Walker et al.(229) found that ENOS (endothelial nitric

oxide synthase) did not correlate with ADPKD severity. Whole genome quantitative trait loci mapping of 114 selected pcy/pcy mice (68 with the mild PKD and 46 with severe PKD) identified two loci, MOP1 and MOP2 that strongly modulate PKD progression (230). Mrug et al. (128) demonstrated that the Kinesin family member 12 gene is a potential candidate modifier gene in *cpk* cystic disease. The results of my study (Chapter 3) provide Cux-1 as a target potential candidate modifier gene for ARPKD. Clinical screening for Cux-1 and the Kinesin family member 12 gene should be performed to evaluate genetic polymorphisms in correlation with disease severity in ARPKD patients.

The next issue became understanding the mechanism behind how *Cux-1ΔCR1/cpk* mice have accelerated PKD. In Chapter 4, I found that Cux-1ΔCR1 is ectopically expressed in proliferating cystic epithelial cells (**Figure 4-1**). In regard to the phenomenon of accelerated PKD occurring within these animals, these results suggested a link between Cux-1ΔCR1 and cell proliferation. It was also a surprising result since mutant Cux-1ΔCR1 carries the same promoter as wild-type Cux-1 (37). During the time of this discovery it was reported that Cux-1 undergoes a degradation pathway that is initiated by the nuclear protease Cathepsin-L (32, 33). Under close examination of the region that was deleted in the Cux-1ΔCR1 mutation, we found that Cux-1ΔCR1 lacks a Cathepsin-L cleavage site (**Figure 4-3**). The absent cleavage site may impair the ability of Cathepsin-L to proteolytically process Cux-1ΔCR1. This in turn could lead to the accumulation of Cux-1ΔCR1 protein which was observed in *cpk* cystic epithelial cells (**Figure 4-1**). Furthermore, I found that

p27 is downregulated in *Cux-1ΔCR1/cpk* kidneys (**Figure 4-5**), where p27 is normally upregulated in *cpk* kidneys (**Figure 1-17**). Therefore, the data I have presented within this dissertation suggests a possible mechanism that helps explain the accelerated PKD observed in *Cux-1ΔCR1/cpk* kidneys (**Figure 5-3**).

The future directions from my studies in Chapter 4 should center on the ability of Cux-1ΔCR1 protein to avoid Cathepsin-L proteolytic cleavage. This can be accomplished by treating purified Cux-1ΔCR1 protein with different concentrations of Cathepsin-L or with the same concentration of Cathepsin-L at different time points in comparison with treatment with wild-type purified Cux-1. Further analysis should involve the transfection of cells with *Cux-1ΔCR1* to determine if overexpression of *Cux-1ΔCR1* results in reduction of p27 promoter luciferase activity and rapid progression through the cell cycle as indicated by Flourescence associated cell sorting (FAC) analysis. However, these *in vitro* results will only confirm or not confirm the *in vivo* data that I have already presented in my dissertation. It would be very unclear if conflicting results were to be observed *in vitro* during these experiments. The data could always be rationalized as being "different" from the naturally occurring events I observed in *vivo*.

The involvement of Cux-1 in ADPKD needs to be confirmed in the same manner by which I have confirmed Cux-1 involvement in ARPKD. I think the overall critical next experiment should be to cross *cux-1ΔCR1* gene onto any selected mouse model for ADPKD where cysts develop over a longer extended period of time

compared to the 1 month period in which cystic disease occurs postnatally in *cpk* mice. The reason why this is important is because we have already established from my results that Cux-1 function seems to be different in ADPKD and ARPKD. Phenotypic alteration of the ADPKD phenotype would confirm the extent and involvement of Cux-1 with the disease which may further help to reveal differences between ADPKD and ARPKD.

The results from this body of work link Cathepsin-L to the ectopic expression of Cux-1 in human and mouse ADPKD as well as mouse ARPKD. The nuclear expression of Cathepsin-L is inversely related to Cux-1 expression in human ADPKD and *pkd1* null mice (**Figure 4-1**, **Figure 4-2** and **Figure 4-3**). That is, there is decreased nuclear Cathepsin-L present when Cux-1 protein levels are upregulated. Cathepsin L isoforms that are devoid of a signal peptide were recently shown to be present in the nucleus where they proteolytically process the CCAAT-displacement protein/cut homeobox (CDP/Cux) transcription factor (32, 33). A role for nuclear Cathepsin L in cell proliferation could be inferred from the observation that the CDP/Cux processed isoform can accelerate entry into S phase (32, 33). In renal carcinoma cells the proteolytic processing of CDP/Cux is augmented and correlates with increased cysteine protease expression and activity in the nucleus (231). Goulet et al.(231) recently suggested that the increased protease activity could be a major factor involved in cell-cycle progression and increased cell proliferation occurring within these cells. They showed that transformation by the *ras* oncogene causes rapid increases both in the production of short nuclear cathepsin L isoforms and in

the processing of CDP/Cux (231). Evidence has linked PKD with cancer (232-234) in that aberrant cell proliferation and growth occurs in both diseases. Therefore, the data within my dissertation strengthens the concept that PKD can be correlated with Cancer in regard to the observation that nuclear Cathepsin-L is highly expressed in PKD and Cux-1 protein appears to be more stable in ADPKD (**Figure 4-2** and **Figure 4-3**).

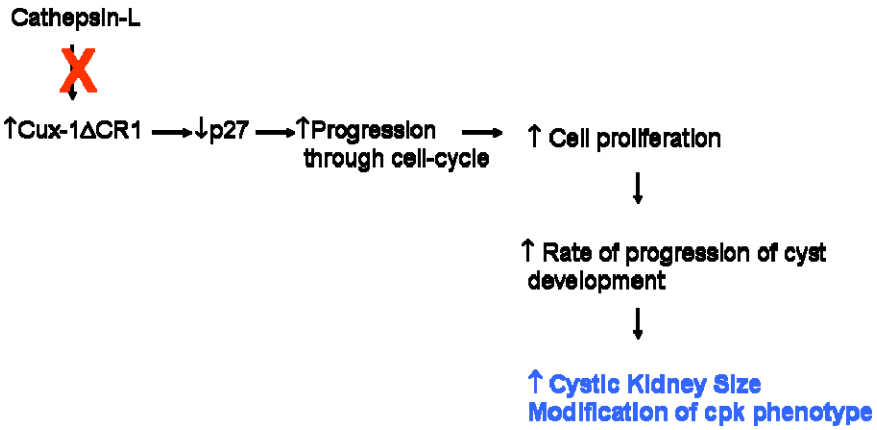


Figure 5-3. Model for accelerated PKD observed in *Cux-1ΔCR1/cpk* kidneys. Since Cux-1ΔCR1 protein lacks a Cathepsin-L cleavage site it may avoid proteolytic degradation and thus accumulate in cystic epithelial cells. Increased Cux-1ΔCR1 protein can bind p27 promoter leading to the reduction of p27 expression levels. Reduced p27 favors rapid progression through the cell cycle resulting in increased numbers of cells dividing and aberrant cell proliferation. Aberrant cell proliferation is a hallmark for cyst progression and development therefore leading to increased advanced stage cyst formation and increased cystic kidney size thus modifying the *cpk* phenotype.

My work has demonstrated that the Cux-1ΔCR1 gain-of-function mutation can exacerbate PKD in the *cpk* mouse and is correlated with downregulation of p27 (**Figure 3-4**, **Figure 3-5**, **Figure 3-6** and **Figure 4-5**). This suggests that Cux-1 can modify PKD. However, the degree by which this mutation of Cux-1 is involved in generating new mitotic signaling pathways and/or altering pre-existing signaling pathways involved in PKD is not clear. It is also conceivable that ectopic Cux-1 expression in cells that were originally differentiated and then undergo dedifferentiation participate in the induction of apoptotic pathways.

Previous studies have suggested that Cux-1 expression may be involved in apoptosis (135, 235). Yet mutant Cux-1ΔCR1 protein is expressed in proliferating cells (**Figure 4-1**) and may help to drive cell proliferation by repressing the cyclin kinase inhibitor p27. However, Cux-1ΔCR1 protein is expressed in cyst-lining epithelial cells undergoing apoptosis at post-natal day 10 (**Figure 4-1**). Cux-1 and Cux-1ΔCR1 ectopic expression occurs in cystic epithelial cells that are undergoing apoptosis at 3 weeks (135) and 10 days of age (**Figure 4-6**) respectively within *cpk* kidneys. Therefore, it appears that the *cux-1ΔCR1/cpk* post-natal day 10 cystic kidneys resemble 3-week old *cpk* kidneys. Caspase expression and activity has been correlated with increased apoptosis occurring within cells (231). Truscott et al. (236) recently found that caspase activity can proteolytically cleave Cux-1 during S-phase and enhance Cux-1 function to accelerate cell-cycle progression. It is possible that the increased activated Caspase-3 protein levels within *cux-1ΔCR1/cpk* mice (**Figure 4-6**) contribute to the acceleration of cystic epithelial cells through the cell cycle

resulting in increased cell proliferation. However, the role of Cux-1 in apoptotic pathways is not clear and it is less clear how apoptosis is involved in PKD. Therefore it may be too early at this stage to draw an analogy between Cux-1 and apoptotic pathways involved in PKD.

Overexpression of Cux-1 in transgenic kidneys results in the downregulation of p27 (20). This observation fits well with the observation that p27 is downregulated in ADPKD and *pkd1* null mice where Cux-1 is upregulated (135). Since Cux-1 targets the cyclin kinase inhibitors p21 (55) and p27 (20) for repression Cux-1 may be involved in downregulation of these cyclin kinase inhibitors in PKD. Bhunia et al. (148) demonstrated that the Polycystin-1 protein signals through the JAK-STAT pathway to upregulate p21. They found that when the *pkd1* gene is mutated, Polycystin-1 signaling through JAK-STAT1 is altered resulting in increased p21. It is possible that the JAK-STAT pathway directly or indirectly regulates Cux-1 transcriptional expression or DNA binding ability and that Cux-1 is a downstream mediator of JAK-STAT signaling (**Figure 5-4**). For example, Cux-1 may be in a phosphorylated state during normal Polycystin-1 JAK STAT signaling resulting in decreased Cux-1 p21 promoter DNA interactions leading to the upregulation of p21. In contrast, defective JAK-STAT signaling could result in the decreased ability of Cux-1 to become phosphorylated in turn triggering Cux-1 DNA binding which could lead to repression of p21 gene expression. Future experiments should target JAK STAT signaling in regard to the expression and phosphorylation state of Cux-1.

Exposure of human epidermal carcinoma (A-431) cells to EGF results in a significant increase in EGFR, Stat-3 phosphorylation and increased p21 cyclin kinase inhibitor expression (237-239). However, inhibition of EGFR by AG1478 fails to decrease EGF-induced Stat-3 phosphorylation (237). This suggests that EGF continues to drive Stat-3 phosphorylation through other receptors. It is interesting to note that p21 is downregulated in A-431 cells that are not stimulated with EGF (238) when Cux-1 protein levels are reduced (Chapter 3). A-431 EGF stimulated cells were similar to that of Polycystin-1 transfected cells from Bhunia et al. (148), whereby stimulation of STAT leads to upregulation of p21 and decreased cell proliferation. This suggests that the JAK STAT pathway, much like in PKD, is active within A-431 cells and may be involved in the regulation of Cux-1 and p21. Therefore, taken together the EGFR pathway and JAK STAT stimulation in addition to Polcystin-1 signaling to JAK STAT may regulate p21 expression through Cux-1 expression level or phosphorylation state (**Figure 5-4**). Whether or not Cux-1 is involved within the JAK STAT pathway still remains to be seen. Transient or stable transfection experiments involving activation of JAK or STAT to determine Cux-1 expression and phosphorylation state in correlation with p21 and/or p27 promoter luciferase activity might address some of those questions.

Since Cux-1 is highly expressed in A-431 cells that are unstimulated with EGF (Chapter 3), it is very attractive to consider that Cux-1 is involved in repression of p21. However, the EGF induced increase in p21 levels in A-431 cells is the result of post-transcriptional and post-translational stabilization (238). Therefore, the

extent of Cux-1 regulation of p21 within these cells is unclear. Moreover, our results indicate that p27 is upregulated in A-431 cells unstimulated with EGF. Cell cycle progression to mitosis in A-431 cells appears to involve p21 but probably not p27, however further experiments are required to confirm or reject this hypothesis. The function of Cux-1 within these cells remains unclear.

EGF elevates intracellular calcium in A-431 cells (239). Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cytosolic calcium mobilized from intracellular stores in A-431 cells. A-431 cells that are not stimulated with EGF highly express Cux-1 (Chapter 3) and undergo cell division in the presence of lower intracellular calcium levels (238, 239). Therefore, there is precedence for Cux-1 expression being regulated by EGFR and associated with reduced intracellular calcium levels and increased cell proliferation. Deficient intracellular calcium and irregular EGFR activity present with aberrant cell proliferation has been demonstrated in PKD cystic epithelial cells (130, 163, 219, 220, 222). Cux-1 is upregulated in cystic epithelial cells (135). Therefore, the involvement of Cux-1 in PKD may be EGFR and calcium dependent as Cux-1 is similarly upregulated under the similar conditions within human epidermal carcinoma cells undergoing cell proliferation.

My results in Chapter 2 implicate Calcineurin in the regulation of Cux-1 by dephosphorylation. Dephosphorylation of Cux-1 has been demonstrated to activate Cux-1 DNA binding activity (55). Activation of the Calcineurin pathway requires increased intracellular Calcium levels. Manzati et al. (240) and others (154, 155)

found that elevated intracellular calcium and PKC activity results in increased cell proliferation in cells that overexpress Polycystin-1 C-terminus. Puri et al. (221) demonstrated that overexpression of the Polycystin-1 C-terminus resulted in activation of the Calcineurin/nFAT pathway. Polycysin-1 has also been linked to regulating increases in intracellular calcium not only through complex with Polycystin-2, a calcium channel, at the cell membrane but also in the release of calcium from the endoplasmic reticulum (reviewed in 98, 101). Therefore, another possible model for Cux-1 involved in PKD signaling is that a Polycystin-1 mediated increase in intracellular calcium leads to activation of Calcineurin which may directly or indirectly lead to Cux-1 dephosphorylation resulting in increased Cux-1 DNA binding activity to repress p27 and aberrant cell proliferation (**Figure 5-4**).

Coqueret el al. (60) found that PKC phosphorylates Cux-1 which could inhibit Cux-1 DNA binding potential. In PKD 85% of all human cases involve mutation in the PKD-1 gene which encodes Polycystin-1. It is possible that altered PKC signaling leads to reduced levels of phosphorylated Cux-1, which would permit DNA binding and repress the p21 and p27 cyclin kinase inhibitors. Transfection studies involving Polycystin-1 overexpression correlated with PKC, Cux-1 and p27 expression and activity need to be conducted to fully address the existence of a Polycystin-1-PKC-Cux-1-p27 pathway.

The role of cAMP in PKD seems to involve induction of cell proliferation in cystic cells (159, 160, 163, 164). cAMP can trigger activation of PKA which translocates into the nucleus and can stimulate activation of the CREB protein (211

for review). The activated CREB protein then binds to a CRE region, and is then bound to by a CBP (CREB binding protein) which coactivates it, allowing it to switch certain genes on or off. Michl et al. (61) demonstrated that cAMP stimulation of PKA phosphorylates Cux-1 resulting in the reduction of Cux-1 DNA binding affinity within fibroblasts. cAMP stimulation of HEK 293T cells transiently transfected with the C-terminus of Polycystin-1 did not show alterations in Cux-1 expression (unpublished observation). However, the phosphorylation state of Cux-1 under these conditions was not evaluated. Therefore, the role of cAMP in regulating Cux-1 in PKD is still unclear.

Nakamura et al. (139) and others demonstrated that TGFβ is upregulated in *cpk* mice. Michl et al. (166, 167) discovered that Cux-1 is a transcriptional target of TGFβ and that Cux-1 activates a transcriptional program regulating genes involved in cell motility, invasion, and extracellular matrix composition. They found that Cux-1 expression is significantly increased in high-grade carcinomas and is inversely correlated with survival in breast cancer. From their results Michl et al. (166, 167) suggested that CUTL1 plays a central role in coordinating a gene expression program associated with cell motility and tumor progression. Therefore, activation of TGFβ in PKD may trigger the upregulation/activation of Cux-1 leading to a "Cancer-like" phenotype in cystic epithelial cells involving increased cell motility and extracellular matrix composition which are characteristics of cystogenesis (233). The involvement of Cux-1 in PKD strengthens the idea that PKD cysts have characteristics similar to cancer/tumor growth.

Results from my dissertation demonstrated that Calcineurin is involved directly or indirectly in Cux-1 dephosphorylation during kidney development (Chapter 2). This was significant because it showed that regulation of Cux-1 phosphorylation state is critical for proper kidney development. I found that the *cux-1ΔCR1* gene accelerates PKD in the *cpk* mouse model for ARPKD establishing a role for Cux-1 in PKD (Chapter 3). This was also an important finding because it identified *cux-1ΔCR1* gene as a candidate modifier gene for ARPKD. The observation that Cux-1ΔCR1 protein is more stable in developing kidneys and ectopically expressed in *cpk* cystic epithelial cells which are simultaneously undergoing cell proliferation and apoptosis led to further insight into possible mechanisms behind accelerated cystogenesis (Chapter 4). Moreover, it led to the observation that nuclear Cathepsin-L levels may help to determine Cux-1 expression in *cpk*, *pkd1* knockout and human ADPKD as a possible explanation for the ectopic expression of Cux-1 in PKD. Further analysis revealed that the cyclin kinase inhibitor p27 is downregulated in *Cux-1ΔCR1/cpk* kidneys which may also help to explain severe PKD occurring within these mice (Chapter 4). This is the fist evidence demonstrating that p27 downregulation can influence PKD severity *in vivo*. Taken together, the results from this dissertation have helped to advance scientific knowledge of kidney development and polycystic kidney disease.

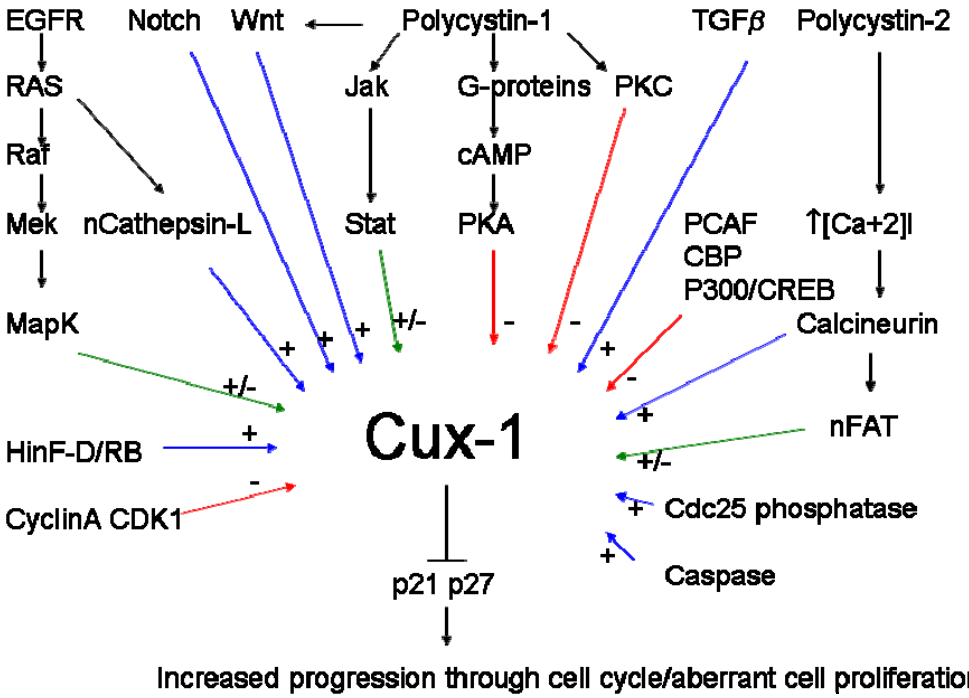


Figure 5-4. Summary of potential signaling pathways that may regulate Cux-1. This model depicts multiple signaling factors/pathways which have been linked to the regulation of Cux-1 within different cell types. Established signaling pathways which occur in polycystic kidney disease are additionally represented in the context of similar factors which have been known to regulate Cux-1 activity (i.e. PKA, PKC) (13, 59-62). The signaling mechanisms described in this model refer to the regulation of Cux-1 by post-translational modification. Proteolytic cleavage (Caspase, Cathepsin-L) and dephosphorylation (Calcineurin pathway, Cdc 25 phosphatase) of Cux-1 favor Cux-1 DNA binding activity which would favor Cux-1 repression of the Cyclin Kinase Inhibitors p21 and p27 (13, 242, 243). The Notch (70), WNT (244) and TGF-β (166) pathways have been demonstrated to correlate with augmented Cux-1 expression levels within different cell types while HinF-D/RB was shown to act as a transcriptional co-repressor complex which involves Cux-1 in mediating transcriptional repression (66, 67). The ability of Cux-1 to interact with co-repressors and to be highly expressed within cells would favor Cux-1 mediated transcriptional repression of its gene targets. Phosphorylation of Cux-1 (PKA, PKC, and Cyclin A CDK1) has been demonstrated to inhibit Cux-1 DNA binding ability (reviewed in 13) which would prevent the ability of Cux-1 to repress p21 and p27. (→) Indicates factors which lead to the expression or post-translational modification of Cux-1 which potentiates Cux-1 DNA binding activity. (→) Indicates pathways which lead to post-translational modification which inhibits Cux-1 function. (→) Indicates factors and pathways which have upstream regulators of Cux-1, yet the mechanism of Cux-1 activity within these pathways is still unclear.

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